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(54) **Title:** METHODS OF SCREENING COMPOUNDS FOR GRK6 MODULATING ACTIVITY

(57) **Abstract:** The present invention relates to methods of treating disease by altering G protein coupled receptor kinase (GRK) 6. This may be done by altering the expression or activity of the protein, for example. The present invention may be used for disease diagnosis, by detecting the expression or activity of GRK6. The present invention relates to a GRK6 deficient mouse, GRK6 splice variants, and methods of use. The present invention also relates to methods of identifying compounds that alter GRK6 activity. The present invention relates to disease treatment by altering GRK6 expression or activity.

Methods of Screening Compounds for GRK6 Modulating Activity

[0001] This application claims priority to U.S.S.N. 60/393,789 filed on July 3, 2002, the contents of which are incorporated by reference in their entirety.

[0002] This work was supported by National Institutes of Health Grants DA-06023, NS-19576, MH-40159, and HL-16037 and therefore the government may have certain rights to the invention.

FIELD OF THE INVENTION

[0003] The invention is in the field of identifying compounds that modulate GRK6 and their use in treating disease.

BACKGROUND

[0004] G protein-coupled receptors (GPCRs) are cell surface proteins that translate hormone or ligand binding into intracellular signals. GPCRs are found in all animals, insects, and plants. GPCR signaling plays a pivotal role in regulating various physiological functions including phototransduction, olfaction, neurotransmission, vascular tone, cardiac output, digestion, pain, and fluid and electrolyte balance. Although they are involved in numerous physiological functions, GPCRs share a number of common structural features. They contain seven membrane domains bridged by alternating intracellular and extracellular loops and an intracellular carboxyl-terminal tail of variable length.

[0005] GPCRs have been implicated in a number of disease states, including, but not limited to: cardiac indications such as angina pectoris, essential hypertension, myocardial infarction, supraventricular and ventricular arrhythmias, congestive heart failure, atherosclerosis, renal failure, diabetes, respiratory indications such as asthma, chronic bronchitis, bronchospasm, emphysema, airway obstruction, upper respiratory indications such as rhinitis, seasonal allergies, inflammatory disease, inflammation in response to injury, rheumatoid arthritis, chronic inflammatory bowel disease, glaucoma, hypergastrinemia, gastrointestinal indications such as acid/peptic disorder, erosive esophagitis, gastrointestinal hypersecretion, mastocytosis, gastrointestinal reflux, peptic ulcer, Zollinger-Ellison syndrome, pain, obesity, bulimia nervosa, depression, obsessive-compulsive disorder, organ malformations (for example, cardiac malformations), neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease, multiple sclerosis, Epstein-Barr infection and cancer.

[0006] The magnitude of the physiological responses controlled by GPCRs is linked to the balance between GPCR signaling and signal termination. The signaling of

GPCRs is controlled by two families of intracellular proteins called G protein-coupled receptor kinases (GRKs) and arrestins. Arrestins bind activated GPCRs, including those that have been agonist-activated and especially those that have been phosphorylated by G protein-coupled receptor kinases (GRKs). Multiple GRK enzymes are found in brain regions, but the relative physiological importance of each GRK to the function of any given neurotransmitter receptor was unclear, and no clear role for GRKs in drug abuse or addiction susceptibility had been demonstrated.

BRIEF DESCRIPTION OF DRAWINGS

[0007] The objects and advantages of the invention will be understood by reading the following detailed description in conjunction with the drawings in which:

[0008] Figure 1 illustrates that GRK6 is present in striatal neurons expressing DARPP-32. Upper-left: Immunofluorescence analysis reveals GRK6 immunoreactivity in the striatal neurons of WT mouse (+0.74 from bregma). Upper-right: Lack of GRK6 immunoreactivity in the striatal neurons of a mouse lacking a functional GRK6 gene (GRK6-KO mouse). Lower-left: DARPP-32 immunoreactivity in the striatal neurons of WT mouse. Lower-right: A color image overlay of the two left panels reveals that GRK6 and DARPP-32 are co-localized in the same neuronal population in the striatum of WT mouse.

[0009] Figure 2 shows the targeted inactivation of the mouse GRK6 gene. Figure 2A is a schematic diagram of the wild type mouse GRK6 gene locus, the GRK6/lox targeting vector, the integrated targeting construct, and the Cre recombinase-deleted GRK6 locus (GRK6-KO). GRK6 exons are shown as open boxes, and numbered from the first coding exon. LoxP sites are shown as filled triangles, and the location of the Southern blot probe as a hatched box. Relevant *Nhe*I restriction sites are indicated. Figure 2B shows the genotyping of targeted GRK6-KO mice. The wild type and GRK6-KO loci were distinguished by triplex PCR amplification. The WT GRK6 locus gives a 460 bp band while the GRK6-KO locus gives a 610 bp band, as indicated. Figure 2C illustrates GRK6 protein expression by Western blotting. Membrane proteins from brainstem and striatum of wild type and GRK6-KO animals were subjected to immunoblotting using an anti-GRK6 antiserum. GRK6-KO homozygote animals exhibit a loss of the 68-kDa immunoreactive band compared to wild type animals (Arrow). The 69-kDa band is a non-specific interaction, since it is present in GRK5 and GRK4 homozygote animals and is not recognized by other GRK6 antiserum.

[0010] Figure 3 illustrates the cocaine supersensitivity in GRK6 mutant mice. Figure 3A shows locomotor response of GRK6 mutant (WT: n=24; GRK6 heterozygous: n=21; GRK6-KO: n=15) mice to cocaine (20 mg/kg, intraperitoneal (i.p.)) administration. GRK6 heterozygous and GRK6-KO mice demonstrate greater locomotor behavior than

their wildtype littermates, and are significantly different from WT controls ($p<0.001$, two-way analysis of variance (ANOVA). Figure 3B is a dose-response curve of the effect of cocaine (10-30 mg/kg, i.p.) on horizontal activity of GRK6-KO, heterozygous and WT mice (n=8-24 per group). Both GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to cocaine ($p<0.001$, two-way ANOVA). Figure 3C illustrates cocaine sensitization in GRK6-KO mice. Mice (WT: n=16; GRK6-KO: n=14) were injected daily with cocaine (20 mg/kg, i.p.) for 5 days and 48 hours after the last injection the animals were challenged with the same dose of the drug. Locomotor activity measurements were performed on days 1 (upper-left) and 7 (upper-right). Two-way ANOVA revealed a significant difference ($p<0.001$) between responses of WT mice in Day 7 vs. Day 1, but no such difference was observed in GRK6-KO mice. In addition, responses in sensitized WT mice (Day 7) were not different from that of GRK6-KO mice either in Day 1 or Day 7. The accumulated distance traveled by mice in the 90 min period after cocaine administration on days 1 and 7 are shown in the lower panel. **$p<0.01$; *** $p<0.001$ vs. WT littermates for the 1st day group (Student's t-test).

[0011] Analysis of accumulated distances over 15 min, 30 min, or 60 min after cocaine administration reveals a significant difference ($p<0.001$) between WT and GRK6-KO mice in Day 1 at any period analyzed, but no such differences were observed between sensitized WT and GRK6-KO mice in Days 1 or 7. In sensitized GRK6-KO mice (Day 7), locomotor responses to cocaine were not enhanced vs. that in Day 1 when 30 min, 60 min or 90 min periods after injection were analyzed. However, analysis of first 15 min period after cocaine revealed a moderate increase in total distance traveled by GRK6-KO mice in Day 7 vs. Day 1 (GRK6-KO, Day 1: 3786±459 cm/15 min; Day 7: 5386±571 cm/15 min, $p<0.05$, Student's t-test; for comparison, distance traveled by WT mice, Day 1: 1686±252 cm/15 min; Day 7: 4077±443 cm/15 min, $p<0.001$, Student's t-test).

[0012] Figure 4 illustrates the enhanced locomotor effects of *d*-amphetamine and ß-phenylethylamine in GRK6 mutant mice. Figure 4A shows a time course of horizontal locomotor response of WT (n=10) and GRK6 mutant (GRK6 heterozygous: n=15; GRK6-KO: n=9) in response to *d*-amphetamine (3 mg/kg, i.p.). GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to *d*-amphetamine. $p<0.001$, two-way ANOVA. Figure 4B shows a time course of horizontal locomotor response of WT (n=6) and GRK6 mutant (GRK6 heterozygous: n=11; GRK6-KO: n=6) mice in response to ß-phenylethylamine (50 mg/kg, i.p.). GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to ß-phenylethylamine. $p<0.001$, two-way ANOVA.

[0013] Figure 5 shows analyses of presynaptic dopamine function in WT and

GRK6-KO mice. Figure 5A illustrates striatal tissue levels of dopamine, 5-HT and their metabolites in GRK6-KO and WT littermate mice measured by HPLC-EC (WT: n=5; GRK6: n=7). Figure 5B shows [^{3}H]-dopamine uptake in striatal synaptosomes from GRK6-KO and WT mice (WT: n=4; GRK6: n=4). Figure 5C illustrates extracellular dopamine levels in the striatum of freely moving mice measured using quantitative low perfusion rate microdialysis (WT: n=6; GRK6: n=9). Figure 5D shows the effect of saline and cocaine (20 mg/kg, i.p.) on extracellular dopamine level in the striatum of freely moving mice. Data are presented as a percentage of the average level of dopamine measured in at least three samples collected before the drug administration. (Saline, WT: n=5; GRK6-KO: n=4; Cocaine, WT: n=7; GRK6-KO: n=6).

[0014] Figure 6 illustrates that alterations in GRK6 level modulate dopamine receptor coupling to G-proteins. Figure 6A shows [^{35}S]GTPγS binding to striatal membranes from GRK6 mutant and wild type mice. Total [^{35}S]GTPγS binding is portrayed after subtracting unstimulated [^{35}S]GTPγS binding from each point. [^{35}S]GTPγS binding to striatal membranes was determined after stimulation with the D2 dopamine agonist quinpirole. Percent stimulated [^{35}S]GTPγS binding was calculated by dividing unstimulated [^{35}S]GTPγS binding into each agonist-stimulated point. Nonlinear regressions were used to calculate the EC_{50} parameters (WT: 2.0±0.5 μM; GRK6-KO: 1.9±0.6 μM). In the absence of agonist stimulation, basal [^{35}S]GTPγS binding did not differ between genotypes. Experiments were performed in triplicate in which WT and GRK6-KO striatal tissue were analyzed simultaneously (n=8 per group). $p<0.001$, two-way ANOVA, GRK6-KO versus WT controls. Figure 6B shows [^{35}S]GTPγS binding to HEK-293 cell membranes expressing the D3 dopamine receptor subtype (D2R) was determined after stimulation with dopamine. At least two independent experiments were performed in triplicate. The same procedure was employed for data treatments. $p<0.001$, two-way ANOVA. Figure 6C shows [^{35}S]GTPγS binding to HEK-293 cell membranes expressing the D3 dopamine receptor subtype (D3R) plus the G protein subunit Go-α was determined after stimulation with dopamine. At least two independent experiments were performed in triplicate. The same procedure was employed for data treatments. $p<0.001$, two-way ANOVA.

[0015] Figure 7 illustrates that the dopamine agonist effect is enhanced in dopamine-depleted GRK6-KO mice. To deplete brain dopamine, animals were treated with a combination of reserpine (5 mg/kg, i.p.) and α-methyl-*p*-tyrosine (250 mg/kg, i.p.). Figure 7A shows a time-course of effect of apomorphine (0.5 mg/kg, s.c.) on the horizontal activity (counts) of dopamine-depleted wild type (n=11) and GRK6-KO (n=7) mice. GRK6-KO mice are significantly different from WT controls ($p<0.001$, two-way ANOVA). Figures 7B and 7C show the dose - response of the effect of apomorphine (0.2-1 mg/kg, s.c.) on the locomotion of dopamine-depleted wild type and mutant mice

(n=6-11 per group). Note that GRK6-KO mice were more affected by apomorphine both in terms of horizontal activity counts (7B) and total distance traveled (7C). $p<0.001$ vs. wild type group for horizontal acitivity counts (7B) and $p<0.05$ for total distance traveled (7C) measurements, two-way ANOVA.

[0016] Figure 8 illustrates that locomotion in DA-depleted wild type and GRK6-KO mice were restored by administration of apomorphine (0.2 mg/kg, s.c.)

[0017] Figure 9 illustrates that locomotion in DA-depleted wild type and GRK6-KO mice were restored by administration of apomorphine (0.5 mg/kg, s.c.).

[0018] Figure 10 shows the nucleic acid and protein sequences of the present invention. SEQ ID No: 1 is the nucleic acid sequence inserted into the pBS vector, as described in Figure 2. SEQ ID No: 2 is the nucleic acid sequence of the Cre-deleted locus, as described in Figure 2. SEQ ID Nos: 3, 4, and 5 are the nucleic acid sequences of the primers used to confirm the construction of the GRK6 deletion. SEQ ID Nos: 6, 8, and 10 are the nucleic acid sequences of the mouse GRK6A, GRK6B, and GRK6c splice variants. SEQ ID Nos: 7, 9, and 11 are the amino acid sequences of the mouse GRK6A, GRK6B, and GRK6c splice variants. SEQ ID Nos: 12, 14, and 16 are the nucleic acid sequences of the human GRK6A, GRK6B, and GRK6c splice variants. SEQ ID Nos: 13, 15, and 17 are the amino acid sequences of the human GRK6A, GRK6B, and GRK6c splice variants.

DETAILED DESCRIPTION

[0019] The present inventors have determined the role of GRK6 in desensitization of GPCRs and have constructed a transgenic mouse that has a functionally disrupted GRK6 gene. The present inventors have determined that GRK6 is a target for modulating desensitization of GPCRs and have designed methods for the identification of compounds that target GRK6 and alter GPCR desensitization. The present inventors describe methods of evaluating the compounds for the treatment of disease and describe the use of such compounds for the treatment of disease.

[0020] In accordance with the present invention there may be employed conventional molecular biology, microbiology, immunology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook *et al*, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (2000)]; "Immobilized

Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984); *Using Antibodies: A Laboratory Manual: Portable Protocol No. I*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1998); *Using Antibodies: A Laboratory Manual*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1999).

[0021] Unless otherwise stated, the following terms used in the specification and claims have the meanings given below:

[0022] A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

[0023] A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

[0024] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (*e.g.*, restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

[0025] An "origin of replication" refers to those DNA sequences that participate in the initiation of DNA synthesis.

[0026] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0027] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[0028] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding

sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

[0029] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[0030] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[0031] The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[0032] The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, *i.e.*, in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[0033] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers

must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[0034] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[0035] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[0036] Two DNA sequences are "substantially homologous" when at least about 65% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g.*, Maniatis *et al.*, supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

[0037] It should be appreciated that also within the scope of the present invention are DNA sequences encoding the same amino acid sequence as SEQ ID NO: 7, 9, 11, 13, 15,or 17, but which are degenerate to SEQ ID NO: 7, 9, 11, 13, 15,or 17. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid.

[0038] "Arrestin" means all types of naturally occurring and engineered variants of

arrestin, including, but not limited to, visual arrestin (sometimes referred to as Arrestin 1), cone arrestin (sometimes referred to as arrestin-4), ß-arrestin 1 (sometimes referred to as Arrestin 2), and ß-arrestin 2 (sometimes referred to as Arrestin 3).

[0039] "ßARK1" is a GRK termed ß-adrenergic receptor kinase 1, also called GRK2.

[0040] "ßAR" is a GPCR termed a ß-adrenergic receptor.

[0041] "Internalization" of a GPCR is the translocation of a GPCR from the cell surface membrane to an intracellular vesicular membrane, where it may be inaccessible to substances remaining outside the cell.

[0042] "Carboxyl-terminal tail" means the carboxyl-terminal tail of a GPCR following membrane span 7. The carboxyl-terminal tail of many GPCRs begins shortly after the conserved NPXXY motif that marks the end of the seventh transmembrane domain (*i.e.* what follows the NPXXY motif is the carboxyl-terminal tail of the GPCR). The carboxyl-terminal tail may be relatively long (approximately tens to hundreds of amino acids), relatively short (approximately tens of amino acids), or virtually non-existent (less than approximately ten amino acids). As used herein, "carboxyl-terminal tail" shall mean all three variants (whether relatively long, relatively short, or virtually non-existent), and may or may not contain palmitoylated cysteine residue(s).

[0043] "Class A receptors" preferably do not translocate together with arrestin proteins to endocytic vesicles or endosomes in association with arrestin-GFP in HEK-293 cells.

[0044] "Class B receptors" preferably do translocate together with arrestin proteins to endocytic vesicles or endosomes associated with arrestin-GFP in HEK-293 cells.

[0045] "DACs" mean any desensitization active compounds. Desensitization active compounds are any compounds that influence the GPCR desensitization mechanism by either stimulating or inhibiting the process. DACs may influence the GPCR desensitization pathway by acting on any cellular component of the process, as well as any cellular structure implicated in the process, including but not limited to: arrestins, GRKs, GPCRs, phosphoinositide 3-kinase, AP-2 protein, clathrin, protein phosphatases, and the like. DACs may include, but are not limited to, compounds that inhibit arrestin translocating to a GPCR, compounds that inhibit arrestin binding to a GPCR, compounds that stimulate arrestin translocating to a GPCR, compounds that stimulate arrestin binding to a GPCR, compounds that inhibit GRK phosphorylation of a GPCR, compounds that stimulate GRK phosphorylation of a GPCR, compounds that stimulate or inhibit GRK binding to a GPCR, compounds that inhibit protein phosphatase dephosphorylation of a GPCR, compounds that stimulate protein phosphatase dephosphorylation of a GPCR, compounds that prevent GPCR internalization or recycling to the cell surface, compounds that regulate the release of

arrestin from a GPCR, antagonists of a GPCR, inverse agonists and the like. DACs may inhibit or stimulate the GPCR desensitization process and may not bind to the same ligand binding site of the GPCR as traditional agonists and antagonists of the GPCR. DACs may act independently of the GPCR, *i.e.*, they do not have high specificity for one particular GPCR or one particular type of GPCRs. DACs may bind the same site(s) as agonist or antagonist but do not desensitize the receptor (perhaps by not altering the receptor to be properly phosphorylated or bind to arrestin or any other protein). DACs may bind to allosteric sites on the receptor and inhibit or enhance desensitization.

[0046] "Detectable molecule" means any molecule capable of detection by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to, fluorescence, phosphorescence, and bioluminescence and radioactive decay. Detectable molecules include, but are not limited to, GFP, luciferase, ß-galactosidase, rhodamine-conjugated antibody, and the like. Detectable molecules include radioisotopes, epitope tags, affinity labels, enzymes, fluorescent groups, chemiluminescent groups, and the like. Detectable molecules include molecules which are directly or indirectly detected as a function of their interaction with other molecule(s).

[0047] "GFP" means Green Fluorescent Protein which refers to various naturally occurring forms of GFP which may be isolated from natural sources or genetically engineered, as well as artificially modified GFPs. GFPs are well known in the art. See, for example, U.S. Patent Nos. 5,625,048; 5,777,079; and 6,066,476. It is well understood in the art that GFP is readily interchangeable with other fluorescent proteins, isolated from natural sources or genetically engineered, including but not limited to, yellow fluorescent proteins (YFP), red fluorescent proteins (RFP), cyan fluorescent proteins (CFP), blue fluorescent proteins, luciferin, UV excitable fluorescent proteins, or any wave-length in between. As used herein, "GFP" shall mean all fluorescent proteins known in the art.

[0048] "Unknown or Orphan Receptor" means a GPCR whose function and/or ligands are unknown.

[0049] "Downstream" means toward a carboxyl-terminus of an amino acid sequence, with respect to the amino-terminus.

[0050] "Upstream" means toward an amino-terminus of an amino acid sequence, with respect to the carboxyl-terminus.

[0051] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site in order to allow formation of disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" residue (*i.e.*, His can act as an acid or

base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces ß-turns in the protein's structure.

[0052] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[0053] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[0054] An "immunoglobulin" includes antibodies and antibody fragments with immunogenic activity. Preferred immunogenic activity is where the immunoglobulin binds to a modified GPCR. An even more preferable immunoglobulin is one that can distinguish between a modified GPCR and a wild-type GPCR. The term "antibody" refers to immunoglobulins, including whole antibodies as well as fragments thereof that recognize or bind to specific epitopes. The term antibody encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. The term "epitope" is used to identify one or more portions of an antigen or an immunogen which is recognized or recognizable by antibodies or other immune system components.

[0055] Exemplary immunoglobulins are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope. Antibody fragments include those portions known in the art as Fab, Fab', $F(ab')_2$, F(v), and scFv
which portions are preferred for use in the therapeutic methods described herein.

[0056] Fab and $F(ab')_2$ portions of antibody fragments are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous *et al.* Fab' antibody portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation with a reagent such as

iodoacetamide. An antibody containing intact antibody portions is preferred herein.

[0057] An "antibody combining site" is that structural portion of an antibody comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0058] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular epitope on an antigen. A monoclonal antibody may therefore contain a plurality of antibody combining sites, each immunospecific for a different antigen, *e.g.*, a bispecific (chimeric) monoclonal antibody.

[0059] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[0060] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce some feature of pathology such as for example, elevated blood pressure, respiratory output, etc.

[0061] A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (*e.g.*, ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[0062] "Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine (A) and thymine (T) are complementary nucleobases that pair through the formation of hydrogen bonds.

[0063] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65 °C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein

hybridization is typically 10-20 °C below the predicted or determined Tm with washes of higher stringency, if desired.

[0064] By "animal" is meant any member of the animal kingdom including vertebrates (*e.g.*, frogs, salamanders, chickens, or horses) and invertebrates (*e.g.*, worms, etc.). "Animal" is also meant to include "mammals." Preferred mammals include livestock animals (*e.g.*, ungulates, such as cattle, buffalo, horses, sheep, pigs and goats), as well as rodents (*e.g.*, mice, hamsters, rats and guinea pigs), canines, felines, primates, lupine, camelid, cervidae, rodent, avian and ichthyes.

[0065] "Antagonist(s)" include all agents that interfere with wild-type and/or modified GPCR binding to an agonist, wild-type and/or modified GPCR desensitization, wild-type and/or modified GPCR binding arrestin, wild-type and/or modified GPCR endosomal localization, internalization, and the like, including agents that affect the wild-type and/or modified GPCRs as well as agents that affect other proteins involved in wild-type and/or modified GPCR signaling, desensitization, endosomal localization, resensitization, and the like.

[0066] "GPCR" means G protein-coupled receptor and includes GPCRs naturally occurring in nature, as well as GPCRs which have been modified. Such modified GPCRs are described in U.S.S.N. 09/993,844 and U.S.S.N. 10/054,616.

[0067] "Abnormal GPCR desensitization" and "abnormal desensitization" mean that the GPCR desensitization pathway is disrupted such that the balance between active receptor and desensitized receptor is altered with respect to wild-type conditions. Either there is more active receptor than normal or there is more desensitized receptor than wild-type conditions. Abnormal GPCR desensitization may be the result of a GPCR that is constitutively active or constitutively desensitized, leading to an increase above normal in the signaling of that receptor or a decrease below normal in the signaling of that receptor.

[0068] "Biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject; wherein said sample can be blood, serum, a urine sample, a fecal sample, a tumor sample, a cellular wash, an oral sample, sputum, biological fluid, a tissue extract, freshly harvested cells, or cells which have been incubated in tissue culture.

[0069] Concurrent administration," "administration in combination," "simultaneous administration," or "administered simultaneously" mean that the compounds are administered at the same point in time or sufficiently close in time that the results observed are essentially the same as if the two or more compounds were administered at the same point in time.

[0070] "Conserved abnormality" means an abnormality in the GPCR pathway, including but not limited to, abnormalities in GPCRs, GRKs, arrestins, AP-2 protein,

clathrin, protein phosphatase and the like, that may cause abnormal GPCR signaling. This abnormal GPCR signaling may contribute to a GPCR-related disease.

[0071] "Desensitized GPCR" means a GPCR that presently does not have ability to respond to agonist and activate conventional G protein signaling.

[0072] "Desensitization pathway" means any cellular component of the desensitization process, as well as any cellular structure implicated in the desensitization process and subsequent processes, including but not limited to, arrestins, GRKs, GPCRs, AP-2 protein, clathrin, protein phosphatases, and the like. In the methods of assaying of the present invention, the polypeptides may be detected, for example, in the cytoplasm, at a cell membrane, in clathrin-coated pits, in endocytic vesicles, endosomes, any stages in between, and the like.

[0073] "GPCR signaling" means GPCR induced activation of G proteins. This may result in, for example, cAMP production.

[0074] "G protein-coupled receptor kinase" (GRK) includes any kinase that has the ability to phosphorylate a GPCR.

[0075] "*Homo sapiens* GPCR" means a naturally occurring GPCR in a *Homo sapiens*.

[0076] "Inverse agonist" means a compound that, upon binding to the GPCR, inhibits the basal intrinsic activity of the GPCR. An inverse agonist is a type of antagonist.

[0077] "Modified GRK" means a GRK modified such that it alters desensitization.

[0078] "Naturally occurring GPCR" means a GPCR that is present in nature.

[0079] "Odorant ligand" means a ligand compound that, upon binding to a receptor, leads to the perception of an odor including a synthetic compound and/or recombinantly produced compound including agonist and antagonist molecules.

[0080] "Odorant receptor" means a receptor protein normally found on the surface of olfactory neurons which, when activated (normally by binding an odorant ligand) leads to the perception of an odor.

[0081] The term "pharmaceutically acceptable carrier," as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent.

[0082] "Primatized antibody" means a recombinant antibody containing primate variable sequences or antigen binding portions, and human constant domain sequences.

[0083] "Sensitized GPCR" means a GPCR that presently has ability to respond to agonist and activate conventional G protein signaling.

[0084] "GRK6" includes GRK6 splice variants, including GRK6a, GRK6b, GRK6c,

and GRK6d and a GRK6, of a human, a primate, a feline, a canine, a porcine, a bovine, a caprine, an ovine, or other animals.

[0085] "Modulation" includes at least an up-regulation or down-regulation of the expression, or an increase or decrease in activity of a protein. Modulation of a protein includes the up-regulation, down-regulation, increase or decrease in activity of a protein or compound that regulates a protein. Modulation also includes the regulation of the gene, the mRNA, or any other step in the synthesis of the protein of interest.

[0086] A "GRK6 related disease" refers to a disease affected by GRK6, particularly GRK6 expression or activity. A GRK6 related disease also includes diseases affected by GPCRs that may be phosphorylated and/or regulated by GRK6, such as the dopamine receptor. Such diseases include Parkinson's, schizophrenia, depression, Tourette Syndrome, and drug-addiction.

[0087] "GRK6-associated desensitization" refers to GPCR desensitization in which GRK6 affects the desensitization. The GRK6 may directly phosphorylate the GPCR, or otherwise affect the desensitization of the GPCR.

[0088] An "overexpressed" protein refers to a protein that is expressed at levels greater than wild-type expression levels.

GPCRs and desensitization

[0089] The exposure of a GPCR to agonist produces rapid attenuation of its signaling ability that involves uncoupling of the receptor from its cognate heterotrimeric G-protein. The cellular mechanism mediating agonist-specific or homologous desensitization is a two-step process in which agonist-occupied receptors are phosphorylated by a G protein-coupled receptor kinases (GRKs) and then bind an arrestin protein.

[0090] It is known that after agonists bind GPCRs, G-protein coupled receptor kinases (GRKs) phosphorylate intracellular domains of GPCRs. After phosphorylation, an arrestin protein associates with the GRK-phosphorylated receptor and uncouples the receptor from its cognate G protein. The interaction of the arrestin with the phosphorylated GPCR terminates GPCR signaling and produces a non-signaling, desensitized receptor.

[0091] The arrestin bound to the desensitized GPCR targets the GPCR to clathrin-coated pits or other cellular machinery for endocytosis (*i.e.*, internalization) by functioning as an adaptor protein, which links the GPCR to components of the endocytic machinery, such as adaptor protein-2 (AP-2) and clathrin. The internalized GPCRs are dephosphorylated and are recycled back to the cell surface desensitized, or are retained within the cell and degraded. The stability of the interaction of arrestin with the GPCR is one factor that dictates the rate of GPCR dephosphorylation, recycling,

and resensitization. The involvement of GPCR phosphorylation and dephosphorylation in the desensitization process has been exemplified in U.S.S.N. 09/933,844, filed November 5, 2001, the disclosure of which is hereby incorporated by reference in its entirety.

[0092] Seven distinct GRK genes are known, named GRK1 through GRK7, that were classified into three distinct groups. GRK6 is a member of the GRK4 subfamily of GRKs, which also contains GRK4 and GRK5. Multiple GRK enzymes are found in brain regions, but the relative physiological importance of each GRK to any given neurotransmitter, prior to the present invention, was unclear.

[0093] Brain dopaminergic transmission is critically involved in numerous vital functions, such as movement control, emotion and affect, and its dysfunction is believed to be central in several pathological conditions, including addiction. Physiological responses to dopamine are controlled by a family of G-protein coupled dopamine receptors (including D1-D5), that are expressed in specific brain areas. Sensitivity of dopamine receptors to endogenous and exogenous ligands is known to be an important modulator of dopamine-related functions in physiology and pathology. Supersensitivity of dopamine signaling has been described in several brain disorders, including addiction. Particularly, it is believed that sensitization, an early biochemical and behavioral manifestation of cellular plasticity leading to addiction, is associated with long-term changes in dopamine receptor responsiveness.

[0094] Dopamine receptors, like other members of the G protein-coupled receptor (GPCR) family, are regulated via activation-dependent phosphorylation by a family of G protein-coupled receptor kinases (GRKs). While several *in vitro* studies were focused on the role of GRKs in dopamine receptor regulation, no data on physiological significance of this regulation and *in vivo* specificity of dopamine receptor/GRK interaction are currently available.

[0095] The present inventors demonstrated that GRK6-deficient mice are supersensitive to the locomotor-stimulating effect of psychostimulants, including cocaine and amphetamine, and displayed little further sensitization to chronic treatment with cocaine. In addition, these mice demonstrated an enhanced coupling of striatal dopamine receptors to G proteins and augmented locomotor response to the dopamine agonist apomorphine in dopamine-depleted animals. The results indicated that postsynaptic dopamine receptors are physiological targets for GRK6, and suggests that these regulatory mechanisms contribute to central dopaminergic supersensitivity observed in drug abuse and other pathological conditions, such as Parkinson's disease. Altering the expression and activity of GRK6 will be useful in treatment of diseases associated with dopamine receptor supersensitivity, such as schizophrenia, depression, Tourette Syndrome, and Parkinson's disease

[0096] This supersensitivity is correlated with an increased coupling of dopamine receptors to G proteins, caused by diminished dopamine receptor desensitization. These results indicate that dopamine receptor regulation by GRK6 plays an important role in setting the basal tone of dopamine signaling in the striatum and that diminished GRK6 function may be a predisposing factor affecting drug sensitivity.

[0097] The decrease in GRK6 levels or activity could enhance the behavioral effects of DA agonists in this animal model of Parkinson's disease. Therefore, these data demonstrate that modulating the amount or activity of GRK6 by either pharmacological or genetic approaches would be useful in Parkinson's disease, to increase the effectiveness of the endogenous dopamine or exogenous dopaminomimetic agents such as L-DOPA.

Knock-out mice and animals

[0098] For use as disease models and to test compounds identified herein, modified GRK6 transgenic and knock-out mice and animals may be produced and utilized. For use as disease models, to test compounds identified herein, and to identify GPCRs phosphorylated by GRK6, transgenic and knock-out mice and animals comprising modified components of the desensitization pathway described herein may be produced and utilized. The animal may, for example, be a mouse or an animal as listed herein. Examples related to knock-out animals are described herein. Certain non-limiting embodiments refer specifically to a knock-out mouse, but are intended to encompass animals as described herein.

[0099] The cells of the mouse containing at least one inactive endogenous GRK6 gene. The mouse may be a complete knockout or homozygous for the inactive endogenous GRK6 gene, or the mouse may be a partial knockout or heterozygous for the inactive endogenous GRK6 gene.

[00100] The knockout mouse may be useful for verification that a compound is in fact a GRK6 modulator. For example, the knockout mouse of the present invention may be used as a model for comparison with wild-type mice that have been treated with a GRK6 modulator. This comparison may be used to verify that the compound administered to the wild-type mice is a GRK6 modulator.

[00101] The knockout mouse may also be useful for verification that a compound is in fact a GRK6 activator or inhibitor. For example, partial knockout mice that have been treated with a GRK6 activator or inhibitor may be used as a model for comparison with wild-type mice and complete knockout mice. This comparison may be used to verify that the compound administered is a GRK6 activator or inhibitor.

[00102] The production of GRK6 knockout mice can be carried out in view of the disclosure provided herein and in light of techniques known to those skilled in the art,

such as described in U.S.S.N. 09/469,554, filed December 22, 1999, U.S. Patents Nos. 5,767,337 to Roses *et al.*; 5,569,827 to Kessous-Elbaz *et al.*; and 5,569,824 to Donehower *et al.* (the disclosures of which are hereby incorporated by reference in their entirety); and A. Harada *et al.*, *Nature* 369, 488 (1994). An example of mice for carrying out the present invention are as disclosed below. Sequences described in Figure 10 include the disrupted GRK6 of the transgenic animal, as well as constructs used in making the transgenic animal.

Methods of testing a compound for ability to modulate GRK6

[00103] The present invention is also related to methods of testing a compound for the ability to modulate GRK6. For example, the test compound may be administered to a wild-type non-human animal; and the locomotor response of the wild-type non-human animal exposed to the compound will be compared to the locomotor response of the non-human transgenic animal that has a disrupted GRK6 gene. Tests of locomotor response would be performed as described in the Examples. Other physical and cellular responses may be monitored, as described in the Examples.

Method of screening for compounds which modulate GRK6-associated desensitization

[00104] The present invention relates to methods of screening for compounds which modulate GRK6-associated desensitization. A cell is provided which includes GRK6 and a GPCR. The cell is contacted with a candidate modulator. The cell is monitored for GRK6-associated desensitization. Methods of monitoring desensitization are described herein, and in U.S.S.N. 09/993,844 filed on November 5, 2001, U.S.S.N. 10/054,616 filed on January 22, 2002, and U.S.S.N. 10/101,235 filed on March 19, 2002, which are hereby incorporated by reference in their entirety. The GRK6-associated desensitization may be monitored by determining the cellular distribution of the GRK6, GPCR, or arrestin in the presence of the compound as compared to the cellular distribution in the absence of the compound. The difference between the cellular distribution of the GRK6, GPCR, or arrestin in the presence or absence of the compound(s) may be correlated to modulation of GRK6 activity.

[00105] The candidate modulator may be a pure compound, or may be a heterogeneous mixture of compounds. The mixture may contain certain compounds that modulate GRK6 and other compounds which do not modulate GRK6. The cellular distribution of the GRK6, GPCR, or arrestin may be determined.

Method of identifying compounds

[00106] The present invention relates to methods of identifying compounds that

modulate GRK6-associated desensitization. A cell is provided which includes GRK6, a GPCR, and an arrestin, wherein one of the molecules is detectably labeled and the GRK6 is overexpressed. The cell is contacted with a candidate modulator. The cellular distribution of the GRK6, GPCR, or arrestin in the presence of the compound is compared to the cellular distribution in the absence of the compound. The difference between the cellular distribution of the GRK6, GPCR, or arrestin in the presence or absence of the compound(s) is correlated to modulation of GRK6 activity. Such methods are described herein, and in U.S.S.N. 09/993,844 filed on November 5, 2001, U.S.S.N. 10/054,616 filed on January 22, 2002, and U.S.S.N. 10/101,235 filed on March 19, 2002, which are hereby incorporated by reference in their entirety.

[00107] In an embodiment, the GRK6 is overexpressed. The labeled molecule may be localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes. The detectable molecule may be a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group. The molecule may be detectably labeled due to its interaction with another molecule, which may be detectably labeled.

[00108] The present invention further relates to methods of inhibiting desensitization of the dopamine receptor in a cell. These methods may include contacting the cell with a compound. The compound may be an antisense oligonucleotide, or another compound as described herein. The antisense oligonucleotide may inhibit expression of a nucleic acid encoding GRK6, or another gene that affects GRK6 activity.

<u>Methods of detection</u>

[00109] Methods of detecting the intracellular location of the detectably labeled arrestin, the intracellular location of a detectably labeled GPCR, the intracellular location of a detectably labeled GRK, or interaction of the detectably labeled molecule with a GPCR or any other cell structure, including for example, the concentration of arrestin, GRK, or GPCR at a cell membrane, colocalization of arrestin with GPCR in endosomes, and concentration of arrestin or GPCR in clathrin-coated pits, and the like, will vary dependent upon the detectable molecule(s) used.

[00110] One skilled in the art readily will be able to devise detection methods suitable for the detectable molecule(s) used. For optically detectable molecules, any optical method may be used where a change in the fluorescence, bioluminescence, or phosphorescence may be measured due to a redistribution or reorientation of emitted light. Such methods include, for example, polarization microscopy, BRET, FRET, evanescent wave excitation microscopy, and standard or confocal microscopy.

[00111] In an embodiment arrestin may be conjugated to GFP and the arrestin-GFP conjugate may be detected by confocal microscopy. In another preferred embodiment,

arrestin may conjugated to a GFP and the GPCR or GRK may be conjugated to an immunofluorescent molecule, and the conjugates may be detected by confocal microscopy. In an additional preferred embodiment, arrestin may conjugated to a GFP and the carboxy-terminus of the GPCR may be conjugated to a luciferase and the conjugates may be detected by bioluminescence resonance emission technology. In a further preferred embodiment arrestin may be conjugated to a luciferase and GPCR may be conjugated to a GFP, and the conjugates may be detected by bioluminescence resonance emission technology. The methods of the present invention are directed to detecting GPCR activity. The methods of the present invention allow enhanced monitoring of the GPCR pathway in real time.

[00112] In an embodiment, the localization pattern of the detectable molecule is determined. In a further preferred embodiment, alterations of the localization pattern of the detectable molecule may be determined. The localization pattern may indicate cellular localization of the detectable molecule. Certain methods of detection are described in U.S.S.N. 10/095,620, filed March 12, 2002, which claims priority to U.S. Provisional Patent Application No: 60/275,339, filed March 13, 2001, the contents of which are incorporated by reference in their entirety.

[00113] Molecules may also be detected by their interaction with another detectably labeled molecule, such as an antibody.

Conjugates

[00114] The cells used in the methods of assaying of the present invention may comprise a conjugate of a GRK protein and a detectable molecule, and the like. The detectable molecule allows detection of molecules interacting with the detectable molecule, as well as the molecule itself.

[00115] All forms of GRKs, naturally occurring and engineered variants, may be used in the present invention. GRKs may interact to a detectable level with all forms of GPCRs.

[00116] Detectable molecules that may be used include, but are not limited to, molecules that are detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to bioluminescence, phosphorescence, and fluorescence. These detectable molecules should be a biologically compatible molecule and should not compromise the biological function of the molecule and must not compromise the ability of the detectable molecule to be detected. Preferred detectable molecules are optically detectable molecules, including optically detectable proteins, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. More preferred detectable molecules are inherently fluorescent

molecules, such as fluorescent proteins, including, for example, Green Fluorescent Protein (GFP). The detectable molecule may be conjugated to the GRK protein by methods as described in Barak *et al.* (U.S. Patent Nos. 5,891,646 and 6,110,693). The detectable molecule may be conjugated at the front-end, at the back-end, or in the middle.

[00117] The GPCRs may also be conjugated with a detectable molecule. Preferably, the carboxyl-terminus of the GPCR is conjugated with a detectable molecule. If the GPCR is conjugated with a detectable molecule, proximity of the GPCR with the GRK may be readily detected. In addition, if the GPCR is conjugated with a detectable molecule, compartmentalization of the GPCR with the GRK may be readily confirmed. The detectable molecule used to conjugate with the GPCRs may include those as described above, including, for example, optically detectable molecules, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. Preferred optically detectable molecules may be detected by immunofluorescence, luminescence, fluorescence, and phosphorescence.

[00118] For example, the GPCRs may be antibody labeled with an antibody conjugated to an immunofluorescence molecule or the GPCRs may be conjugated with a luminescent donor. In particular, the GPCRs may be conjugated with, for example, luciferase, for example, Renilla luciferase, or a rhodamine-conjugated antibody, for example, rhodamine-conjugated anti-HA mouse monoclonal antibody. Preferably, the carboxyl-terminal tail of the GPCR may be conjugated with a luminescent donor, for example, luciferase. The GPCR, preferably the carboxyl-terminal tail, also may a be conjugated with GFP as described in L. S. Barak *et al.* Internal Trafficking and Surface Mobility of a Functionally Intact ß2-Adrenergic Receptor-Green Fluorescent Protein Conjugate, Mol. Pharm. (1997) 51, 177 - 184.

<u>**Cell types and substrates**</u>

[00119] The cells of the present invention may express at least one GRK, and GPCR, wherein at least one of the molecules is detectably labeled. Cells useful in the present invention include eukaryotic and prokaryotic cells, including, but not limited to, bacterial cells, yeast cells, fungal cells, insect cells, nematode cells, plant cells, and animal cells. Suitable animal cells include, but are not limited to, HEK cells, HeLa cells, COS cells, and various primary mammalian cells. An animal model expressing a conjugate of a GRK6 and a detectable molecule throughout its tissues or within a particular organ or tissue type, may also be used in the present invention.

[00120] A substrate may have deposited thereon a plurality of cells of the present invention. The substrate may be any suitable biologically substrate, including but not

limited to, glass, plastic, ceramic, semiconductor, silica, fiber optic, diamond, biocompatible monomer, or biocompatible polymer materials.

Expression of proteins

[00121] Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host.

[00122] Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

[00123] A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g., E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAS, *e.g.*, the numerous derivatives of phage λ, *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

[00124] Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the lac system, the trp system, the TAC system, the TRC system, the LTR system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (*e.g.*, Pho5), the promoters of the yeast α-mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[00125] A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*,

fungi such as yeasts, plant cells, nematode cells, and animal cells, such as HEK-293, CHO, RI.I, B-W and L-M cells, African Green Monkey kidney cells (*e.g.*, COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (*e.g.*, Sf9), and human cells and plant cells in tissue culture.

[00126] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

[00127] In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, *e.g.*, their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

[00128] Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

[00129] It is further intended that modified GRK6 analogs may be prepared from nucleotide sequences of the protein complex/subunit derived within the scope of the present invention. Analogs, such as fragments, may be produced, for example, by pepsin digestion of GRK6 material. Other analogs, such as muteins, can be produced by standard site-directed mutagenesis of GRK6 coding sequences. Analogs exhibiting "GRK6 activity" such as small molecules, whether functioning as promoters or inhibitors, may be identified by known *in vivo* and/or *in vitro* assays.

[00130] As mentioned above, a DNA sequence encoding a modified GRK6 can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the GRK6 amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by

standard methods and assembled into a complete coding sequence. See, *e.g.*, Edge, Nature, 292:756 (1981); Nambair *et al.*, Science, 223:1299 (1984); Jay *et al.*, J. Biol. Chem., 259:6311 (1984).

[00131] Synthetic DNA sequences allow convenient construction of genes which will express GRK6 analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native or modified GRK6 genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis.

[00132] A general method for site-specific incorporation of unnatural amino acids into proteins is described in Christopher J. Noren, Spencer J. Anthony-Cahill, Michael C. Griffith, Peter G. Schultz, Science, 244:182-188 (April 1989). This method may be used to create analogs with unnatural amino acids.

Method of evaluating treatments of GRK6-associated disease

[00133] Provided in the present invention are methods of evaluating treatments of GRK6-associated disease. The compound that modulates GRK6 will be administered to a wild-type non-human animal. The locomotor responses of this animal will be compared to the locomotor responses of the transgenic animal with a functionally disrupted GRK6 gene. Means of examining the locomotor responses are described in the examples.

Method of treating or diagnosing a disease

[00134] The present invention is related to methods of treating or diagnosing a disease. The disease treatment may involve administering a compound that modulates GRK6. The compound may directly or indirectly modulate GRK6. The compound may be an antisense molecule or an immunoglobulin. The disease may by Parkinson's disease, schizophrenia, depression, Tourette Syndrome, or drug-addiction. The methods of disease diagnosis relate to the detection of the GRK6 protein, nucleic acid, or activity in a sample. Such methods include detection using immunoglobulins, nucleic acids, and antisense molecules.

[00135] The methods of disease treatment of the present invention include the concurrent administration of the compound that modulates GRK6 with an additional compound. The additional compound may directly or indirectly affect dopamine levels. Such compounds include L-DOPA, cocaine, and morphine. The compound that modulates GRK6 may increase the effectiveness of the additional compound. The concurrent administration of the compound that modulates GRK6 may decrease the amount of the additional compound required by the patient.

Disease treatment

[00136] The present invention relates to methods of treating a human or non-human subject suffering from a GPCR-related disease, such as cardiovascular disease, heart failure, asthma, nephrogenic diabetes insipidus, hypertension, Parkinson's disease, schizophrenia, depression, Tourette Syndrome, or drug-addiction. Such treatment can be performed either by administering to a subject in need of such treatment, an amount of the compound identified by the present method sufficient to treat the GPCR-related disease, or at least to lessen the symptoms thereof.

[00137] Treatment may also be effected by administering to the subject the naked modified nucleic acid sequences of the invention, such as by direct injection, microprojectile bombardment, delivery via liposomes or other vesicles, or by means of a vector which can be administered by one of the foregoing methods. Gene delivery in this manner may be considered gene therapy. Preferably, the naked modified nucleic acid sequences comprise modified GRK6 proteins of the present invention.

Diagnostic and Therapeutic Treatments

[00138] The possibilities of both diagnostic and therapeutic treatments that are raised by the existence of the GPCR derive from the fact that the factors appear to participate in direct and causal protein-protein interaction between a ligand thereto, and those factors that thereafter initiate an intracellular signal. As discussed earlier and elaborated further on herein, the present invention contemplates pharmaceutical intervention in the cascade of reactions in which the GRKs or GPCRs are implicated, to modulate the activity initiated by the GRK.

[00139] Thus, in instances where it is desired to reduce or inhibit the activity resulting from a particular stimulus or factor, an appropriate inhibitor of the GRK could be introduced to block the phosphorylation of the GPCR by the GRK. Correspondingly, instances in which insufficient activation of a G protein or second messenger is taking place could be remedied by introduction of additional quantities of the GRK or its chemical or pharmaceutical cognates, analogs, fragments and the like. Instances in which excess activation of a G protein or second messenger is taking place could be remedied by introduction of decreased quantities of the GRK or its chemical or pharmaceutical cognates, analogs, fragments and the like.

[00140] The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in a mixture, a pharmaceutically acceptable excipient (carrier) and a compound that modulates a GRK, as described herein as an active ingredient. In an embodiment, the composition comprises a drug capable of modulating the phosphorylation of the GPCR by a GRK.

Pharmaceutical compositions

[00141] The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents that enhance the effectiveness of the active ingredient.

[00142] A GRK6 modulating compound obtained by the methods disclosed herein can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[00143] The therapeutic compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent (*i.e.*, carrier, or vehicle).

[00144] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compositions lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any composition used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range which includes the IC50 (*i.e.*, the concentration of the test composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more

accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00145] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of modulation of GPCR activity desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.001 to 30, preferably about 0.01 to about 25, and more preferably about 0.1 to 20 milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

[00146] The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to, the severity of the disease or condition, disorder, or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the composition(s) can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with the composition in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of the composition used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

[00147] The therapeutic compositions may further include an effective amount of the GRK6 modulating compound and one or more of the following active ingredients: an antibiotic, a steroid, and the like.

[00148] The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention can be prepared as SATE ((S-acetyl-2-thioethyl) phosphate) derivatives according to the methods disclosed for example in WO 93/24510 and in WO 94/26764.

[00149] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. The compounds for modulating any of the disclosed genes, gene transcripts or proteins encoded thereby include antisense compounds as well as other modulatory compounds.

[00150] Pharmaceutically acceptable base addition salts for use with antisense as well as other modulatory compounds are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, *e.g.*, Berge *et al.*, "Pharmaceutical Salts," J. Pharma. Sci., 1977, 66: 1-19). The base addition salts of acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are known in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids (*e.g.*, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid); with organic carboxylic, sulfonic, sulfo- or phospho- acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with

the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

[00151] Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

[00152] For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

[00153] The antisense compounds and other modulatory compounds described herein can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound or other modulatory compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the compounds and methods of the invention may also be useful prophylactically.

[00154] The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding a gene identified using the systematic discovery technique or a mRNA transcript thereof. Such hybridization allows the use of sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding a gene or gene transcript identified by a systematic discovery method can be detected by means known in the art. Such means may include, for example, conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of a transcript of a gene in a sample may also be prepared.

[00155] The present invention also includes pharmaceutical antisense compositions and formulations which include the antisense compounds and other modulatory compounds and compositions of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated.

[00156] In certain embodiments, it may be desirable to administer the pharmaceutical

compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

[00157] For topical application, the compositions may be combined with a carrier so that an effective dosage is delivered, based on the desired activity.

[00158] Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

[00159] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer, salts, flavoring, coloring and sweetening agents as appropriate.

[00160] Preparations for oral administration may be suitably formulated to give controlled release of the active composition.

[00161] The compositions may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as

suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[00162] For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray, presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the composition and a suitable powder base such as lactose or starch.

[00163] The compositions may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compositions may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[00164] The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[00165] Pharmaceutical compositions (*e.g.*, gene, gene transcript or protein product modulatory agents as described herein) of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

[00166] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[00167] In one embodiment of the present invention, the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and

liposomes. While basically similar in nature, these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

[00168] The compositions of the present invention may be prepared and formulated as emulsions. See, *e.g.*, Idson, in Pharmaceutical Dosage Forms v. 1, p. 199 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York); Rosoff, in Pharmaceutical Dosage Forms, v. 1, p. 245; Block in Pharmaceutical Dosage Forms, v. 2, p. 335; Higuchi *et al.*, in Remington's Pharmaceutical Sciences 301 (Mack Publishing Co., Easton, Pa., 1985). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

[00169] Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms v. 1, p. 199 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York).

[00170] Synthetic surfactants, also known as surface active agents, have found wide

applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in Pharmaceutical Dosage Forms,v. 1, p. 285; Idson, in Pharmaceutical Dosage Forms, v. 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in Pharmaceutical Dosage Forms).

[00171] Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers, especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, non-swelling clays (*e.g.*, bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate), pigments and nonpolar solids (*e.g.*, carbon or glyceryl tristearate).

[00172] A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, v.1 p.385 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York)).

[00173] Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers, such as polysaccharides (*e.g.*, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (*e.g.*, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (*e.g.*, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

[00174] Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used

may be free radical scavengers (*e.g.*, tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene) or reducing agents (*e.g.*, ascorbic acid and sodium metabisulfite), and antioxidant synergists (*e.g.*, citric acid, tartaric acid, and lecithin).

[00175] The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in Pharmaceutical Dosage Forms, v. 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in Pharmaceutical Dosage Forms, v. 1, p. 245 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York); Idson, in Pharmaceutical Dosage Forms). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

[00176] In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in Pharmaceutical Dosage Forms, v. 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in Controlled Release of Drugs: Polymers and Aggregate Systems, 185-215 (Rosoff, M., Ed., 1989, VCH Publishers, New York). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, 271 (Mack Publishing Co., Easton, Pa., 1985).

[00177] Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with co-surfactants. The co-surfactant, usually a short-chain alcohol such

as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules.

[00178] Microemulsions may, however, be prepared without the use of co-surfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono-, di-, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

[00179] Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et al.*, Pharm. Res., 1994, 11:1385-90; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13: 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, 1994; Ho *et al.*, J. Pharm. Sci., 1996, 85: 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids and other active agents from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids and other active agents within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

[00180] Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories—surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Crit. Rev. Therap.

Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

[00181] There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, are useful because of their specificity and the duration of action. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

[00182] Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*. Selection of the appropriate liposome depending on the agent to be encapsulated would be evident given what is known in the art.

[00183] In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome that is highly deformable and able to pass through such fine pores.

[00184] Further advantages of liposomes include: (a) liposomes obtained from natural phospholipids are biocompatible and biodegradable; (b) liposomes can incorporate a wide range of water and lipid soluble drugs; (c) liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

[00185] Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

[00186] Another embodiment also contemplates the use of liposomes for topical administration. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin. Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the

targeting of the upper epidermis.

[00187] Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes that interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, Biochem. Biophys. Res. Comm., 1987, 147:980-985).

[00188] Liposomes that are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, J. Controlled Release, 1992, 19: 269-74).

[00189] Another contemplated liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

[00190] "Sterically stabilized" liposomes, which refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids are also contemplated. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside GM1, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, FEBS Lett., 1987, 223: 42; Wu *et al.*, Can. Res., 1993, 53: 3765).

[00191] Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. See, *e.g.*, Sunamoto *et al.* (Bull. Chem. Soc. Jpn., 1980, 53: 2778) described liposomes

comprising a nonionic detergent, 2C12 15G, that contains a PEG moiety. Illum *et al.* (FEBS Lett., 1984, 167: 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (FEBS Lett., 1990, 268: 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029: 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 BI and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by, *e.g.*, Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*). Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

[00192] Methods of encapsulating nucleic acids in liposomes is also known in the art. See, WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Pat. No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes.

[00193] Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, p.285 (Marcel Dekker, Inc., New York, N.Y., 1988, p. 285)).

[00194] If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values

range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

[00195] If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

[00196] If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

[00197] If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

[00198] The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, 285 (Marcel Dekker, Inc., New York, N.Y., 1988).

[00199] In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids and other agents, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

[00200] Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

[00201] Another embodiment of the invention contemplates pharmaceutical

compositions comprising surfactants. Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, Crit. Rev. Therap. Drug Carrier Systems, 1991, 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40: 252).

[00202] Another embodiment contemplates the use of various fatty acids and their derivatives to act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C1-10 alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, and the like) (Lee *et al.*, 1991; Muranishi, Crit. Rev. Therap. Drug Carrier Systems, 1990, 7: 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44: 651-4).

[00203] The compositions comprising the active agents of the invention may further comprise bile salts. The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, N.Y., 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, 1991; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, 1990; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263: 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79: 579-83).

[00204] The invention further contemplates compositions comprising chelating agents. Chelating agents can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers for use when the active agent is an antisense agent, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618: 315-39). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee *et al.*, 1991; Muranishi, 1990; Buur *et al.*, J. Control Rel., 1990, 14: 43-51).

[00205] The invention also contemplates pharmaceutical compositions comprising active agents and non-chelating non-surfactants. Non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants, but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, 1990). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, 1991); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39: 621-6).

[00206] For pharmaceutical compositions comprising oligonucleotides, agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

[00207] Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes (*e.g.*, limonene and menthone).

[00208] Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The

coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, Antisense Res. Dev., 1995, 5: 115-121; Takakura *et al.*, Antisense & Nucl. Acid Drug Dev., 1996, 6: 177-183).

[00209] The pharmaceutical compositions disclosed herein may also comprise a excipients. In contrast to carrier compounds described above, these excipients include a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids or other active agents to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid or other active agent and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (*e.g.*, starch, sodium starch glycolate, etc.); and wetting agents (*e.g.*, sodium lauryl sulphate, etc.).

[00210] Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

[00211] Formulations for topical administration of nucleic acids and other contemplated active agents may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic

acids or other contemplated active agents can be used.

[00212] The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, *e.g.*, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

[00213] Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[00214] In another related embodiment, compositions of the invention may contain one or more antisense compound or other active agents. Two or more combined compounds may be used together or sequentially.

Antisense

[00215] The present invention further includes the preparation of antisense oligonucleotides and ribozymes that may be used to interfere with the expression of GRK6, and the like at the translational level. Preferably, the antisense and ribozymes may be used to interfere with the expression of a GRK6, and the like having discrete point mutations that increases its affinity for arrestin in suspect target cells. This approach utilizes antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

[00216] Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. (See Weintraub, Sci Am. 1990 Jan;262(1):40-6; Marcus-Sekura, Anal Biochem. 1988 Aug 1;172(2):289-95). In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly

efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into cells. Antisense methods have been used to inhibit the expression of many genes *in vitro* (Marcus-Sekura, 1988; Hambor *et al.*, J Exp Med. 1988 Oct 1;168(4):1237-45).

[00217] Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, Gene. 1988 Dec 20;73(2):259-71). Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

[00218] Investigators have identified two types of ribozymes, Tetrahymena-type and "hammerhead"-type. (Hasselhoff and Gerlach, 1988) Tetrahymena-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to Tetrahymena-type ribozymes for inactivating a specific mRNA species, and eighteenbase recognition sequences are preferable to shorter recognition sequences.

[00219] The DNA sequences described herein may thus be used to prepare antisense molecules against, and ribozymes that cleave mRNAs for GRK6s and their ligands. In particular, the antisense molecules and ribozymes may be particularly useful for GRK6s having mutations that alter their affinity for GPCRs.

Antibodies

[00220] The invention further provides antibodies, preferably monoclonal antibodies, which specifically bind to the polypeptides of the invention. Methods are also provided for producing antibodies in a host animal. The methods of the invention comprise immunizing an animal with at least one GRK6 -derived immunogenic component, wherein the immunogenic component comprises one or more of the polypeptides encoded by any one of SEQ ID NO: 1 - SEQ ID NO: 3 or sequence-conservative or function-conservative variants thereof; or polypeptides that are contained within any ORFs, including complete protein-coding sequences, of which any of SEQ ID NO: 1 - SEQ ID NO: 3 forms a part; or polypeptide sequences contained within any of SEQ ID NO: 1 - SEQ ID NO: 3; or polypeptides of which any of SEQ ID NO: 1 - SEQ ID NO: 3 forms a part. Host animals include any warm blooded animal, including without limitation mammals and birds. Such antibodies have utility as reagents for

immunoassays to evaluate the abundance and distribution of GRK6 antigens.

[00221] Antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of GRK6 and/or their biologically active fragments or subunits may possess certain diagnostic or therapeutic applications. For example, the GRK6a, GRK6b, GRK6c, GRK6d or fragments or subunits thereof may be used to produce both polyclonal and monoclonal antibodies, to GRK6 or subunits thereof, in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic or antagonize the activity(ies) of the GRK6 of the invention may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols. Fragments of the GRK6 sequence may be prepared synthetically as peptides and covalently conjugated to a carrier protein (such as Keyhole limpet hemocyanin) or fused directly to the coding region of a carrier protein (such as glutathione S-transferase) and expressed as a unit. Such carrier conjugated are injected into the host species to allow antibodies to be produced. This method was employed to make an antibody against the last 30 unique residues of the GRK6B splice variant.

[00222] The present invention likewise extends to the development of antibodies against GRK6, including naturally raised and recombinantly prepared antibodies. For example, the antibodies could be used to screen expression libraries to obtain the gene or genes that encode subunits of the GRK6. Such antibodies could include both polyclonal and monoclonal antibodies prepared by known genetic techniques, as well as bi-specific (chimeric) antibodies, and antibodies including other functionalities suiting them for additional diagnostic use conjunctive with their capability of modulating GRK6 activity. Preferably, the anti-GRK6 antibody used in the diagnostic methods of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-modified-GPCR antibody fragments used herein be in the form of Fab, Fab', $F(ab')_2$, F(v), or scFv.

[00223] The general methodology for making monoclonal antibodies by hybridomas is well known. Methods for producing monoclonal anti-GRK6 antibodies are also well-known in the art. See Niman *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:4949-4953 (1983). Typically, the GRK6 or a peptide analog is used either alone or conjugated to an immunogenic carrier, as the immunogen in the before described procedure for producing anti-GRK6 monoclonal antibodies. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibodies into the medium. The hybridomas are screened for the ability to produce an antibody that immunoreacts with the GRK6 or peptide analog. The antibody-containing medium is then collected. The antibody can then be further isolated by well-known techniques.

Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, *e.g.*, *Using Antibodies: A Laboratory Manual*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1999).

[00224] Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco *et al.*, *Virol.* 8:396 (1959)) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. A preferred inbred mouse strain is the Balb/c.

[00225] Methods for producing polyclonal anti-polypeptide antibodies are well-known in the art. See U.S. Patent No. 4,493,795 to Nestor *et al.* A monoclonal antibody, and immunologically active fragments thereof, can be prepared using the hybridoma technology described in *Using Antibodies: A Laboratory Manual*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1999), which is incorporated herein by reference. Briefly, to form the hybridoma from which the monoclonal antibody composition is produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunized with a GRK6. Splenocytes are typically fused with myeloma cells using polyethylene glycol (PEG) 6000 MW. Fused hybrids are selected by their sensitivity to HAT (hypoxanthine, aminopterin, thymidine) supplemented media. Hybridomas producing a monoclonal antibody useful in practicing this invention are identified by their ability to immunoreact with the present GRK6 and their ability to inhibit specified GRK6 activity in target cells.

EXAMPLES

Example 1

Targeted deletion of the mouse GRK6 locus

[00226] The three Triple-Lox vectors described for the GRK5 knockout were modified to have new multiple cloning sites, and phage ë carrying fragments of the mouse GRK6 gene from the 129/SvJ strain were obtained and sequenced as described. The targeting vector (Figure 2A) contained the 7-kb *Nhe*I-*Not*I fragment (exons 10-15), loxP site, 2.75-kb *Xba*I-*Nhe*I fragment (exons 3 through 9), loxP site, TK-NEO marker gene cassette, loxP site, and 1.3-kb *Xba*I gene fragment (exon 2).

[00227] Growth and selection of targeted ES cells and creation of chimeric mice was performed as described in B. Hogan, *et al*, Manipulation of the Mouse Embryo: A Laboratory Manual, 2nd Edition (Cold Spring Harbor, NY: Harbor Press, 1994). The targeting DNA was linearized by digestion with *Not*I, and electroporated into AK7 ES cells. A targeted cell clone with a normal karyotype was expanded and microinjected

into day 3.5 C57BL/6J mouse blastocysts, which were then injected into the uterus of a day 2.5 pseudopregnant B6SJLF1/J mouse. Chimeric founders were crossed with C57BL/6J mice to generate agouti pups that carried the targeted 'lox' GRK6 gene. F1 heterozygote animals were bred with transgenic mice bearing CMV-Cre (backcrossed to a C57Bl/6J genetic background to induce deletion of the floxed cassettes. From offspring of these crosses, GRK6 knockout (GRK6-KO) animals were obtained, in which both the exon 3-9 cassette and the TK-NEO marker gene cassettes were deleted. Deletion of exons 3 through 9 leads to a GRK6 that lacks most of the amino terminal RGS-like domain as well as half of the conserved catalytic domain elements (*i.e.*, the gene is inactive) (Fig 2A). Genotyping was routinely performed on tail tip DNA using a PCR method utilizing three primers to simultaneously detect the wild type and mutant loci (Figure 2B).

[00228] Cell culture and transfection: HEK293 cells (ATCC, Rockville, MD) were cultured in MEM supplemented with 10% fetal calf serum and penicillin/streptomycin at 37°C under 5% CO_2/95% air. Cells are transfected with appropriate expression plasmid DNAs using calcium phosphate co-precipitation.

[00229] Western Blots. Mouse brain regions were dissected on ice and immediately frozen in liquid nitrogen. Crude membranes were prepared from mouse brain regions by polytron homogenization in buffer (20mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.4) followed by centrifugation at 200 x g for 2 min and then at 21,000 x g for 30 min. Aliquots (60 μg) of each sample were solubilized by addition of SDS-PAGE sample buffer and separated by 10% SDS-PAGE. Transferred proteins blotted with polyclonal anti-GRK6 and visualized using enhanced chemiluminescent development (ECL, Amersham, Piscataway, NJ).

[00230] Immunohistochemistry. Wild type or GRK6-KO mice (n = 3) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4 °C). Brains were post-fixed for 1-3 days and cryoprotected in 10% sucrose. Free floating coronal or saggital sections of 25 μm were incubated 32 h at 4 °C with a mixture of antibodies against DARPP-32 (mouse, 1:5000, D97520, BD Transduction laboratories, K) and GRK6 (rabbit, 1:50, sc-566, Santa Cruz Biotechnologies, Inc.). The second immunoreaction step was performed by incubation (1 h at room temperature) each of the following antibodies: fluorescein-isothiocyanate-labelled goat antirabbit IgG and Texas-Red-labelled goat anti-mouse IgG (Vector laboratories). Slides were viewed on a laser scanning Zeiss confocal microscope (LSM-510) using the Roper Scientific Cooled CCD digital camera (Coolsnap-FX, BioVision Technologies, Inc, PA) and the IPLab software for Windows v3.0 for image processing (BioVision Technologies, Inc). Images were acquired separately in each channel (dual scan mode) to eliminate the

possibility of signal bleed-over from one channel to the other.

[00231] Animal treatment/drugs/behavior. 3-4 month old littermate wild type (WT) and GRK6 mutant mice (C57BL/6J x 129/SvJ) were used in these experiments. In all experiments, wild type littermates served as controls for mutant mice, all the genotypes were evaluated concurrently, and each animal was used in only a single test. Horizontal and vertical activities of littermate wild type, heterozygote and knockout mice of both genders were measured in an Omnitech Digiscan activity monitor (42 cm^2). Locomotor activity was measured at 5 min intervals and cumulative counts were taken for data analysis. To evaluate the effects of cocaine, morphine and ß-phenylethylamine on locomotor behavior, mice were placed in activity monitor, 30-60 min later they were injected with drugs or vehicle i.p., and locomotor activity was monitored for the following 90 min. In cocaine sensitization experiments, mice were treated chronically with cocaine (20 mg/kg, i.p.) for 5 days and their responses to challenging dose of cocaine were analyzed at day 7. To analyze the effect of direct dopamine agonist in dopamine-depleted mice, animals were pre-treated with a combination of reserpine (5 mg/kg, i.p. 20 h before the experiment) and ß-methyl-*p*-tyrosine (250 mg/kg, i.p., 1h before the experiment). This treatment resulted in depletion of striatal dopamine to less than 0.75% in both wild type and GRK6-KO mice. Mice were completely immobilized by this treatment. Dopamine-depleted mice were treated with vehicle or D1/D2 dopamine receptor agonist apomorphine (0.2-1 mg/kg, s.c.) and locomotor activity was immediately analyzed as described above. In all acute experiments, each animal received only a single injection with a tested drug. All the data presented in this study are expressed as means ± SEM.

[00232] Neurochemical assessments. For monoamine analyses, brain regions were dissected, monoamines extracted, and analyzed for levels of dopamine, serotonin (5-hydroxytryptamine, 5-HT) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) using high performance liquid chromatography with electrochemical detection as described. To perform *in vivo* microdialysis experiments, mice were anesthetized and dialysis probes were implanted into the right striatum. Twenty four hours after surgery, the dialysis probe was connected to a syringe pump and perfused with artificial CSF. Quantitative "low perfusion" rate (70 nl/min) microdialysis experiments were conducted in freely moving mice for determination of basal extracellular dopamine levels in striatum. To analyze the effects of cocaine on the extracellular dopamine levels in striatum, "conventional" microdialysis method (perfusion flow rate 1 μl/min) in freely moving animals was employed.

[00233] To measure [^{3}H]-dopamine uptake in striatal synaptosomes, striatal tissue from wild type and GRK6-KO mice were homogenized in a sucrose buffer (0.32M

sucrose, 4.2mM Hepes, pH 7.4). Homogenates were centrifuged at 1,500 x g for 15 min and supernatants were collected and re-centrifuged at 10,000 x g for 15 min. The resulting pellets were washed and resuspended in buffer (0.02 % ascorbic acid, 50 μM pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose; pH 7.4). Synaptosomal samples were incubated at 37 °C for 2 min with 20 nM of [^{3}H]-dopamine (31.6 Ci/mmol). Non-specific uptake was carried out in the presence of 5 μM mazindol. Incubations were then stopped by adding 3 ml cold wash buffer (50 mM Tris-HCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, pH 7.4) and vacuum-filtered through glass microfiber filters. The filters were then washed 2 times with cold wash buffer and placed in vials containing scintillation cocktail.

[00234] Analyses of dopamine receptor coupling by [^{35}S]GTPγS binding *in vivo* and *in vitro*. In *in vivo* experiments, D2/D3 dopamine receptor agonist quinpirole-stimulated [^{35}S]GTPγS binding to striatal membranes from GRK6-KO and wild type mice was assessed as previously described. To directly assess *in vitro* the role of GRK6 in D2 and D3 dopamine receptor regulation, dopamine-stimulated [^{35}S]GTPγS binding to cultured cell membranes was used. HEK-293 cells were transfected with D2R or D3R/Goα with and without GRK6. For D2R, 20 μg of cell membrane proteins were incubated in a buffer containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 3 μM GDP, and 0.1 nM [^{35}S]GTPγS for 1 hr at room temperature. For D3R, 20 μg of cell membrane proteins were incubated in a buffer containing 25 mM HEPES, pH 7.4, 120 mM NaCl, 1.8 mM KCl, 20 mM $MgCl_2$, 20 μM GDP, 0.2 nM [^{35}S]GTPγS, and 1 mM sodium deoxycholate for 2 hr at 30 °C. Incubation mixtures were filtered with GF/B filter and washed with 10 mM sodium phosphate buffer.

Example 2

GRK6 expressed in neuronal populations containing a key dopaminergic signaling molecule

[00235] To understand the role of GPCR desensitization mechanisms in sensitization to drugs of abuse, we have begun to examine mice bearing inactivated GRK genes for alterations in cocaine responses and dopamine receptor function. Previous histological examinations have shown that GRK6 mRNA is expressed in many brain regions, including primary dopaminergic areas, such as substantia nigra as well as dorsal and ventral striatum. The expression level of GRK6 mRNA in the striatum was found to be higher than that of other GRKs (GRK2, GRK3 and GRK5), suggesting that GRK6 might be a predominant receptor kinase in this brain area. A detailed investigation of the expression pattern of GRK6 protein using immunohistochemistry revealed expression of this kinase in the majority of cells in both dorsal and ventral striatum (Fig. 1). Particularly, GRK6 protein was found in the same neuronal population that expresses

DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein, apparent molecular weight of 32,000 Da), a key molecule involved in dopaminergic signaling mediated both by D1-like and by D2-like dopamine receptors, and a phenotypic marker of the medium-size spiny GABA neurons of the mammalian striatum (Fig. 1). These neurons represent a major striatal cell group receiving dopaminergic input and are believed to be critically involved in cellular mechanisms of addiction. In addition, dense expression of GRK6 protein was detected in a population of large-sized aspiny cholinergic interneurons, which represent another major group of dopaminoceptive striatal cells.

[00236] Figure 1 illustrates that GRK6 is present in striatal neurons expressing DARPP-32. Upper-left: Immunofluorescence analysis reveals GRK6 immunoreactivity in the striatal neurons of WT mouse (+0.74 from bregma). Upper-right: Lack of GRK6 immunoreactivity in the striatal neurons of GRK6-KO mouse. Lower-left: DARPP-32 immunoreactivity in the striatal neurons of WT mouse. Lower-right: GRK6 and DARPP-32 are co-localized in the same neuronal population in the striatum of WT mouse. GRK6 immunoreactivity was detected using a commercially available anti-GRK6 antibody (rabbit, 1:50, sc-566, Santa Cruz Biotechnologies, Inc.). Similar observations were made using another anti-GRK6 antiserum. Note that GRK6 is also expressed in large cholinergic striatal cells, which do not express DARPP-32, but can be labeled with anti - choline acetyltransferase antibody. Scale bar is equal 50 μm.

Example 3

Method of analyzing dopamine-induced locomotor response of GRK6 transgenic mouse as compared to wild-type mouse

[00237] The mouse GRK6 gene was targeted by homologous recombination in embryonic stem cells (Fig. 2A-C). The heterozygote and homozygote GRK6-KO mice are viable and present no gross anatomical or behavioral abnormalities, although GRK6-KO mice demonstrate reduced lymphocyte chemotaxis. In locomotor activity tests, unchallenged knockout mice were not different from wild type littermates either in horizontal (Fig.3A,B) or vertical activities. However, acute cocaine (20 mg/kg, i.p.) administration resulted in a markedly enhanced locomotor response in GRK6 mutant mice (Fig. 3A,B). In this paradigm, the GRK6-KO mice exhibited a more pronounced and longer lasting locomotor activation, as measured by horizontal (Fig. 3A,B) and vertical activities in response to cocaine (10-30 mg/kg, i.p.) than did wild type littermate mice. Interestingly, the mice heterozygous for GRK6 deletion were as responsive to cocaine as GRK6 "null" mice (Fig. 3A,B), suggesting that even minor changes in GRK6 levels or activity may result in significant behavioral alterations. The degree of activation induced by cocaine (20 mg/kg, i.p.) in both heterozygous and knockout mice was substantially higher, not only relative to littermate wild type mice, but also relative to mice of both parental strains (C57BL/6J and 129/SvJ) used to generate the mutants.

Importantly, no such supersensitivity to cocaine was observed in GRK5-KO mice.

[00238] Figure 2. Targeted inactivation of the mouse GRK6 gene. A. Schematic diagram of the wild type GRK6 locus, the GRK6/lox targeting vector, the integrated targeting construct, and the Cre recombinase-deleted GRK6 locus (GRK6-KO). GRK6 exons are shown as open boxes, and numbered from the first coding exon. LoxP sites are shown as filled triangles, and the location of the Southern blot probe as a hatched box. Relevant NheI restriction sites are indicated. B. Genotyping of targeted GRK6-KO mice. The wild type and GRK6-KO loci were distinguished by triplex PCR amplification as described in Methods. The WT GRK6 locus gives a 460 bp band while the GRK6-KO locus gives a 610 bp band, as indicated. C. GRK6 protein expression by Western blotting. Membrane proteins from brainstem and striatum of wild type and GRK6-KO animals were subjected to immunoblotting using an anti-GRK6 antiserum. GRK6-KO homozygote animals exhibit a loss of the 68-kDa immunoreactive band compared to wild type animals (Arrow). The 69-kDa band is a non-specific interaction, since it is present in GRK5 and GRK4 homozygote animals and is not recognized by other GRK6 antiserum.

[00239] Figure 3. Cocaine supersensitivity in GRK6 mutant mice. A. Locomotor response of GRK6 mutant (WT: n=24; GRK6 heterozygous: n=21; GRK6-KO: n=15) mice to cocaine (20 mg/kg, i.p.) administration. GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to cocaine ($p<0.001$, two-way analysis of variance (ANOVA). B. Dose-response curve of the effect of cocaine (10-30 mg/kg, i.p.) on horizontal activity of GRK6-KO, heterozygous and WT mice (n=8-24 per group). Both GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to cocaine ($p<0.001$, two-way ANOVA). C. Cocaine sensitization in GRK6-KO mice. Mice (WT: n=16; GRK6-KO: n=14) were injected daily with cocaine (20 mg/kg, i.p.) for 5 days and 48 hours after the last injection animals were challenged with the same dose of the drug. Locomotor activity measurements were performed on days 1 (upper-left) and 7 (upper-right). Two-way ANOVA revealed a significant difference ($p<0.001$) between responses of WT mice in Day 7 vs. Day 1, but no such difference was observed in GRK6-KO mice. In addition, responses in sensitized WT mice (Day 7) were not different from that of GRK6-KO mice either in Day 1 or Day 7. The accumulated distance traveled by mice in the 90 min period after cocaine administration on days 1 and 7 are shown in the lower panel. **$p<0.01$; *** $p<0.001$ vs. WT littermates for the 1st day group (Student's t-test). Analysis of accumulated distances over 15 min, 30 min, or 60 min after cocaine administration reveals a significant difference ($p<0.001$) between WT and GRK6-KO mice in Day 1 at any period analyzed, but no such differences were observed between sensitized WT and GRK6-KO mice in Days 1 or 7. In sensitized GRK6-KO mice (Day

7), locomotor responses to cocaine were not enhanced vs. that in Day 1 when 30 min, 60 min or 90 min periods after injection were analyzed. However, analysis of first 15 min period after cocaine revealed a moderate increase in total distance traveled by GRK6-KO mice in Day 7 vs. Day 1 (GRK6-KO, Day 1: 3786±459 cm/15 min; Day 7: 5386±571 cm/15 min, $p<0.05$, Student's t-test; for comparison, distance traveled by WT mice, Day 1: 1686±252 cm/15 min; Day 7: 4077±443 cm/15 min, $p<0.001$, Student's t-test).

Example 4

Response of wild-type and GRK6 transgenic animals to repeated cocaine administration

[00240] Repeated administration of cocaine is known to result in a progressive enhancement of psychomotor responses. This phenomenon, termed "behavioral sensitization" or "reverse tolerance" is believed to relate to neuronal adaptations associated with drug addiction. In experimental animals it is often modeled by analyzing locomotor responses to repeated intermittent treatments with the same dose of cocaine. To test whether the GRK6-KO animals can be further sensitized to cocaine, such a cocaine-sensitization paradigm was employed. Mice received daily injections of cocaine for 5 consecutive days and were tested for their responses to this drug on the 7th day. Compared to the 1st day of treatment, wild type animals exhibited enhanced locomotor responses (~ 2 fold) to cocaine on day 7 (Fig. 3C). By contrast, GRK6-KO animals were as responsive to cocaine on the 1st day as were wild type mice following the sensitization protocol (Fig. 3C). As might be expected, GRK6-KO mice were substantially less affected by this sensitization regimen. In fact, analysis of total distance traveled for 90 min did not reveal significant differences between day 1 and day 7 (Fig. 3C). Nonetheless, in the first 15 min after cocaine administration, sensitized GRK6-KO mice did exhibit a slightly enhanced response (Fig. 3C, legend). Taken together, these data imply that in the absence of pharmacological treatment, the GRK6-KO mice may already be essentially "pre-sensitized" to cocaine.

Example 5

GRK6 modulation enhanced response to increased dopamine

[00241] The locomotor responses of GRK6 transgenic mice were enhanced in the presence of increased dopamine, induced by amphetamine and ß-phenylethylamine. It is well established that the locomotor stimulating action of cocaine is mediated by the blockade of the dopamine transporter (DAT) and the resultant elevation of extracellular dopamine in the striatum and related brain areas. Another psychostimulant known to markedly enhance central dopaminergic transmission via complex interaction with the DAT is amphetamine. Similarly to cocaine, amphetamine-induced locomotor activation was significantly enhanced in both GRK6 heterozygous and "null" mice, (Fig. 4A).

Furthermore, enhanced locomotor responses, both in heterozygous and homozygous GRK6 mutant mice, were observed when the endogenous "trace amine" ß-phenylethylamine was administered (Fig. 4B). While the functions and mechanism of the stimulant action of ß-phenylethylamine have not been fully determined, it is believed that ß-phenylethylamine primarily acts as an "endogenous amphetamine" via DAT-mediated efflux of dopamine from intraneuronal stores to extracellular spaces. Accordingly, *in vivo* microdialysis experiments revealed that ß-phenylethylamine (50 mg/kg, i.p.) induced potent, but transient elevation in striatal extracellular dopamine to the same degree (6-fold) in both GRK6-KO and wild type mice. Furthermore, no locomotor activation and corresponding rise in extracellular dopamine was observed in mice lacking the DAT. Thus, an enhanced locomotor response of GRK6 mutants to ß-phenylethylamine is also consistent with an enhanced responsiveness to dopaminergic activation.

[00242] Figure 4. Enhanced locomotor effects of *d*-amphetamine and ß-phenylethylamine in GRK6 mutant mice. A. Time course of horizontal locomotor response of WT (n=10) and GRK6 mutant (GRK6 heterozygous: n=15; GRK6-KO: n=9) in response to *d*-amphetamine (3 mg/kg, i.p.). GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to *d*-amphetamine. $p<0.001$, two-way ANOVA. B. Time course of horizontal locomotor response of WT (n=6) and GRK6 mutant (GRK6 heterozygous: n=11; GRK6-KO: n=6) mice in response to ß-phenylethylamine (50 mg/kg, i.p.). GRK6 heterozygous and GRK6-KO mice are significantly different from WT controls in responses to ß-phenylethylamine. $p<0.001$, two-way ANOVA.

Example 6

GRK6-transgenic mice did not differ from wild-type mice in measured neurochemical parameters

[00243] Behavioral supersensitivity to psychostimulants could be explained either by alterations in presynaptic dopaminergic function in these mice leading to augmented extracellular dopamine levels or by altered postsynaptic receptor responsiveness. To test the status of striatal presynaptic dopaminergic transmission in mutant mice, a set of neurochemical approaches was used (Fig. 5). GRK6-KO mice were not different from wild type controls in any of the neurochemical parameters examined. In particular, tissue dopamine and metabolite content, synaptosomal dopamine uptake rates, and basal and cocaine-stimulated extracellular dopamine levels as assessed by *in vivo* microdilaysis were not affected in mutant mice (Fig. 5A-D). Thus, pronounced locomotor supersensitivity to psychostimulants in the GRK6 mutant occurs without measurable alterations in presynaptic dopamine function.

[00244] Figure 5. Analyses of presynaptic dopamine function in WT and GRK6-KO mice. A. Striatal tissue levels of dopamine, 5-HT and their metabolites in GRK6-KO and WT littermate mice measured by HPLC-EC (WT: n=5; GRK6: n=7). B. [^{3}H]-dopamine uptake in striatal synaptosomes from GRK6-KO and WT mice (WT: n=4; GRK6: n=4). C. Extracellular dopamine levels in the striatum of freely moving mice measured using quantitative low perfusion rate microdialysis (WT: n=6; GRK6: n=9). D. Effect of saline and cocaine (20 mg/kg, i.p.) on extracellular dopamine level in the striatum of freely moving mice. Data are presented as a percentage of the average level of dopamine measured in at least three samples collected before the drug administration. (Saline, WT: n=5: GRK6-KO: n=4; Cocaine, WT: n=7; GRK6-KO: n=6).

Example 7

GRK6 modulation results in dopamine receptors that are more efficiently coupled to their G proteins

[00245] To assess potential changes in basal dopamine receptor sensitivity in the absense of GRK6, an analysis of dopamine receptor coupling to G proteins using a [^{35}S]GTPγS binding assay was performed. Direct assessment of striatal dopamine receptor coupling using the D2/D3 dopamine receptor agonist, quinpirole, revealed that in GRK6-KO mice, these receptors are more efficiently coupled to their G proteins (Fig. 6A).

[00246] Thus, this suggests that under basal conditions in the intact animals, dopamine receptors are tonically inhibited by GRK6 action, and the loss of this inhibition in GRK6-KO animals leads directly to receptor supersensitivity (higher coupling). Importantly, ligand binding studies failed to demonstrate any genotypic differences in D2-like dopamine receptor ([^{3}H]-raclopride) binding (Bmax in WT: 123±18, in GRK6-KO: 111±19 fmol/mg protein). Additionally, Gα protein ($G_{i/o/z}$) levels in GRK6-KO mice were not altered, as assessed by immunoblotting assay. To confirm the role of GRK6 in the regulation of dopamine receptors in an *in vitro* system, GRK6 was co-expressed with either D2 or D3 dopamine receptors (D2R or D3R) in HEK293 cells and analysis of dopamine-stimulated [^{35}S]GTPγS binding was performed. In agreement with *in vivo* observations, substantially impaired G protein coupling was observed when these receptors were co-expressed with GRK6 (Fig. 6B,C). Furthermore, co-expression of GRK6 enhanced the basal (unstimulated) translocation of ß-arrestin2 to D2R or D3R. Thus, in an *in vitro* system, GRK6 appears to induce a basal level of desensitization of D2/D3 dopamine receptors that is associated with increased ß-arrestin2 binding. This is in agreement with many previous studies that have shown in other receptor systems that membrane-associated GRK6 induces basal (activation-independent) receptor phosphorylation, and suggests that this basal

receptor phosphorylation tone is physiologically important, at least for the dopamine receptors.

[00247] Figure 6. Alterations in GRK6 level modulate dopamine receptor coupling to G-proteins. A. [^{35}S]GTPγS binding to striatal membranes from mutant and wild type mice. Total [^{35}S]GTPγS binding is portrayed after subtracting unstimulated [^{35}S]GTPγS binding from each point. [^{35}S]GTPγS binding to striatal membranes was determined after stimulation with quinpirole. Percent stimulated [^{35}S]GTPγS binding was calculated by dividing unstimulated [^{35}S]GTPγS binding into each agonist-stimulated point. Nonlinear regressions were used to calculate the EC_{50} parameters (WT: 2.0±0.5 μM; GRK6-KO: 1.9±0.6 μM). In the absence of agonist stimulation, basal [^{35}S]GTPγS binding did not differ between genotypes. Experiments were performed in triplicate in which WT and GRK6-KO striatal tissue were analyzed simultaneously (n=8 per group). $p<0.001$, two-way ANOVA, GRK6-KO versus WT controls. B. [^{35}S]GTPγS binding to HEK-293 cell membranes expressing D2R was determined after stimulation with dopamine. At least two independent experiments were performed in triplicate. The same procedure was employed for data treatments. $p<0.001$, two-way ANOVA. C. [^{35}S]GTPγS binding to HEK-293 cell membranes expressing D3R/Goα was determined after stimulation with dopamine. At least two independent experiments were performed in triplicate. The same procedure was employed for data treatments. $p<0.001$, two-way ANOVA.

Example 8

GRK6 modulation enhances the behavioral effects of dopamine: Dopamine receptor responsiveness is enhanced in GRK6 mutant mice

[00248] Striatal D2/D3 dopamine receptors have been localized both on presynaptic dopamine nerve terminals and postsynaptic striatal cells. To directly assess postsynaptic dopamine receptor responsiveness in GRK6-KO mice, the effects of the non-selective dopamine agonist apomorphine were tested in dopamine-depleted mice where no endogenous dopamine neurotransmission was present (Fig.7). Notably, the paradigm used was originally developed as an animal model to test drugs effective in Parkinson's disorder, where profound loss of dopamine innervation occurs. Wild type and GRK6-KO mice were treated with reserpine (5 mg/kg, i.p.) to deplete intraneuronal storage of monoamines including dopamine, and with α-methyl-*p*-tyrosine (250 mg/kg, i.p.) to inhibit dopamine synthesis (20h and 1h before the experiment, respectively). Both mutant and control mice were completely immobilized by this treatment. Locomotion in dopamine-depleted wild type and mutant mice was restored by administration of mixed D1/D2 dopamine receptor agonist apomorphine (0.2-1 mg/kg, s.c.) (Fig.7A-C). GRK6-KO mice showed a markedly enhanced locomotor response to

apomorphine in comparison to wild type litteramates, directly demonstrating that postsynaptic dopamine receptor responsiveness is enhanced in GRK6 mutant mice. Furthermore, these data suggest that a decrease in GRK6 levels or activity could enhance the behavioral effects of dopamine agonists in this animal model.

[00249] Figure 7. Dopamine agonist effect is enhanced in dopamine-depleted GRK6-KO mice. To deplete brain dopamine, animals were treated with a combination of reserpine (5 mg/kg, i.p.) and α-methyl-*p*-tyrosine (250 mg/kg, i.p.) as described in Materials and Methods. A. Time-course of effect of apomorphine (0.5 mg/kg, s.c.) on the horizontal activity (counts) of dopamine-depleted wild type (n=11) and GRK6-KO (n=7) mice. GRK6-KO mice are significantly different from WT controls ($p<0.001$, two-way ANOVA). B. and C. Dose - response of the effect of apomorphine (0.2-1 mg/kg, s.c.) on the locomotion of dopamine-depleted wild type and mutant mice (n=6-11 per group). Note that GRK6-KO mice were more affected by apomorphine both in terms of horizontal activity counts (B) and total distance traveled (C). $p<0.001$ vs. wild type group for horizontal acitivity counts (B) and $p<0.05$ for total distance traveled (C) measurements, two-way ANOVA.

Example 9

GRK6 modulation increased effectiveness of compounds to treat Parkinson's disease

[00250] To directly assess postsynaptic dopamine (DA) receptor responsiveness in GRK6-KO mice, the effects of the non-selective DA agonist apomorphine were tested in DA-depleted mice where no endogenous neurotransmission was present. The paradigm used was developed by A. Carlsson as a model to test drug effectiveness in parkinsonism, such as the dopamine precursor L-DOPA or direct dopamine receptor agonists such as apomorphine (Carlsson *et al.*, 1991). Mice (n=7-12 per group) were treated with reserpine (5 mg/kg, i.p.) to deplete intraneuronal storage of monoamines including DA, and with α-methyl-p-tyrosine (250 mg/kg, i.p.) to inhibit DA synthesis (24h and 1h before the experiment, respectively). Mice were completely immobilized by this treatment. Locomotion in DA-depleted wild type mice was restored by administration apomorphine (0.2 and 0.5 mg/kg, s.c.) (Fig.8 and Fig. 9). Both GRK6-KO and heterozygous mice showed a markedly enhanced locomotor response to apomorphine ($p<0.01$ vs. wild type group for both 0.2 and 0.5 mg/kg, s.c. apomorphine), suggesting that the decrease in GRK6 levels or activity could enhance the behavioral effects of DA agonists in this animal model of Parkinson's disease. Therefore, these data demonstrate that modulating the amount or activity of GRK6 by either pharmacological or genetic approaches would be useful in Parkinson's disease, to increase the

effectiveness of the endogenous dopamine or exogenous dopaminomimetic agents such as L-DOPA.

[00251] Taken together, these results indicate that postsynaptic D2/D3 dopamine receptors are physiological targets of GRK6 and that supersensitivity to agonist stimulation may occur in striatal neurons in the absence of GRK6. It should be considered, however, that presynaptic D2/D3 dopamine "autoreceptors" and/or other subtypes of dopamine receptors might also be affected in GRK6-KO mice. In addition, the widespread expression of GRK6 in brain suggests that multiple receptor types may be physiological targets for this kinase; detailed investigation will be required to establish the portfolio of receptors affected in GRK6 mutant mice.

[00252] In the present study, we find that direct regulation of dopamine receptor by one of the GPCR specific kinases, GRK6, represents an important determinant by which receptor sensitivity and responses to drugs of abuse can be controlled. The observation that single allele inactivation of GRK6 in mice produces a phenotype identical to the complete knockout of the gene raises the possibility that even subtle allelic variations in the human GRK6 gene or altered GRK6 activity might contribute to individual sensitivity to psychostimulants and other drugs affecting dopaminergic function. Furthermore, a role for GRK6-mediated dopamine receptor regulation in other brain disorders associated with dopamine dysfunction would be of interest to consider. Particularly, a potential of these findings for the development of novel treatment strategies for Parkinson's disease is noteworthy.

[00253] While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

[00254] The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The following documents, as well as any documents referenced in the foregoing text, should be considered as incorporated by reference in their entirety.

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CLAIMS

We Claim:

1. A non-human transgenic animal, whose somatic or germ line cells comprise a disrupted GRK6 gene, wherein the animal exhibits a decrease in G-protein coupled receptor desensitization relative to a wild-type animal.

2. The animal of claim 1, wherein the animal is a mouse.

3. The animal of claim 1, wherein the non-human transgenic animal is a primate, a feline, a canine, a porcine, a bovine, a caprine, or an ovine.

4. A method of testing a compound for the ability to modulate GRK6 activity, comprising: (a) administering the compound to wild-type non-human animal; and (b) comparing the locomotor response of the wild-type non-human animal exposed to the compound to the locomotor response of the non-human transgenic animal of claim 1.

5. A method of screening for modulators of GRK6-associated desensitization comprising: (a) providing a cell comprising a GRK6 and a GPCR; (b) contacting said cell with a candidate modulator; and (c) monitoring said cell for GRK6-associated desensitization.

6. The method of claim 5, wherein the monitoring comprises determining the cellular distribution of the GRK6, GPCR, or arrestin.

7. A method of evaluating treatments of GRK6-associated disease comprising: (a) providing a non-human animal comprising GRK6 and a GPCR; (b) treating said animal with a preselected treatment; (c) exposing said animal to agonist; and (d) monitoring locomotor response of said animal as compared to locomotor response of the transgenic non-human animal of claim 1.

8. A method for identifying compounds that modulate GRK6 comprising the steps of:
(a) providing a cell comprising GRK6, a GPCR, and an arrestin, and wherein at least one of said molecules is detectably labeled;
(b) exposing the cell to the compound(s);
(c) determining the cellular distribution of the GRK6, GPCR, or arrestin;
(d) comparing the cellular distribution of the GRK6, GPCR, or arrestin in the presence

of the compound(s) to the cellular distribution of the GRK6, GPCR, or arrestin in the absence of the compound(s); and

(e) correlating a difference between (1) the cellular distribution of the GRK6, GPCR, or arrestin in the presence of the compound(s) to (2) the cellular distribution of the GRK6, GPCR, or arrestin in the absence of the compound(s) to modulation of GRK6 activity.

9. The method of claim 8, wherein the GRK6 is overexpressed.

10. The method of claim 8, wherein the labeled molecule is localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles or endosomes.

11. The method of claim 8, wherein the detectable molecule is a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group.

12. The method of claim 8, wherein the molecule is detectably labeled due to its interaction with another molecule, which may be detectably labeled.

13. A method for inhibiting desensitization of the dopamine receptor in cell comprising contacting the cell with a compound that decreases GRK6 activity or the expression of a nucleic acid encoding GRK6.

14. The method of claim 13, wherein the compound is an antisense oligonucleotide.

15. The method of claim 14, wherein the antisense oligonucleotide inhibits expression of a nucleic acid encoding GRK6.

16. A method for treating diseases involving the dopamine receptor, wherein the effectiveness of endogenous dopamine is increased by altering GRK6 activity or expression.

17. The method of claim 7, wherein the disease is Parkinson's, schizophrenia, Tourette Syndrome, depression, or drug-addiction.

18. A method of modulating desensitization of a dopamine receptor in a cell, comprising:

(a) providing a cell expressing a dopamine receptor and a G protein coupled receptor kinase (GRK);

(b) modulating the activity of the GRK; and
(c) exposing said cell to an agonist.

19. The method of claim 18, wherein the GRK is GRK6.

20. The method of claim 19, wherein the expression of GRK6 is increased.

21. The method of claim 19, wherein the expression of GRK6 is decreased.

22. The method of claim 19, wherein the activity of GRK6 is increased.

23. The method of claim 19, wherein the activity of GRK6 is decreased.

24. A method of treating a disease by modulating desensitization of a dopamine receptor in a host cell, comprising: (a) providing a compound which modulates the expression or activity of a GRK6; and (b) administering said compound to a host.

25. The method of claim 24, wherein said method comprises concurrent of the compound that modulates expression or activity of a GRK6 with a compound that modulates a G-protein coupled receptor.

26. A nucleic acid selected from the group consisting of SEQ ID Nos: 1-3.

27. A nucleic acid selected from the group consisting of SEQ ID Nos: 4-5.

28. A vector comprising the nucleic acid of SEQ ID Nos:1-3.

29. The vector of claim 28, wherein the nucleic acid is flanked by loxP sites.

30. A host cell comprising the nucleic acid of SEQ ID No:19.

31. An isolated immunoglobulin which recognizes and binds to a GRK, or fragment thereof.

32. The immunoglobulin of claim 31, wherein the GRK6 is GRK6a, GRK6b, GRK6c, or GRK6d.

33. The immunoglobulin of claim 31, wherein the GRK fragment has the sequence of

SEQ ID No. 3.

34. The immunoglobulin of claim 31, wherein the antibody fragment is Fab, Fab', F(ab')2, F(v), and scFv.

35. A method of detecting GRK6 in a biological sample, comprising:
(a) exposing the biological sample to an immunoglobulin of claim 31; and
(b) determine whether the immunoglobulin bound a protein of the biological sample.

36. The method of 35, wherein the binding of the immuno globulin to the protein indicates the presence of or predis-position to a disease.

37. A method of modulating a cell comprising a GRK6 gene, said method comprising the step of introducing into said cell an isolated polynucleotide according to claim 26, whereby the function and/or structure of the GRK6 gene is modulated.

38. A method according to claim 37, wherein the isolated polynucleotide is a knock-out or knock-in construct.

Fig 1

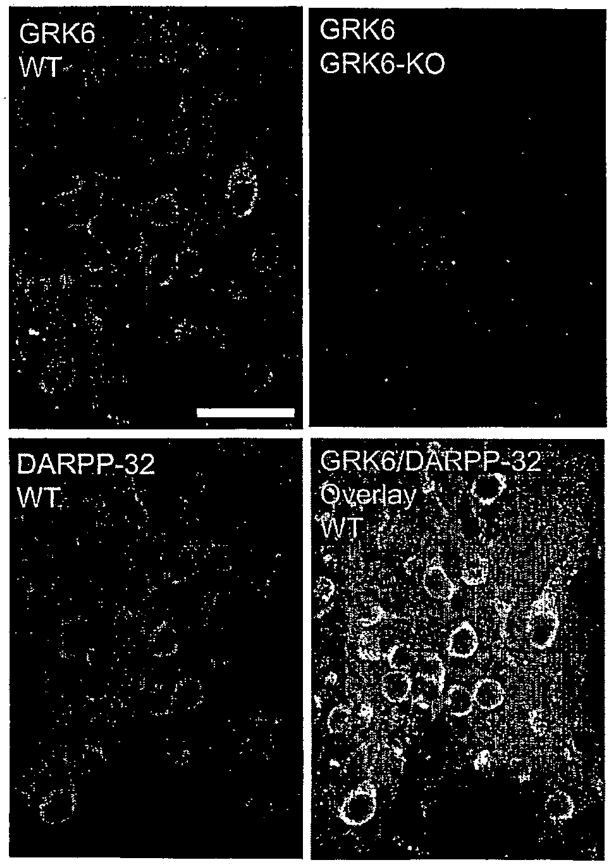


Fig 2

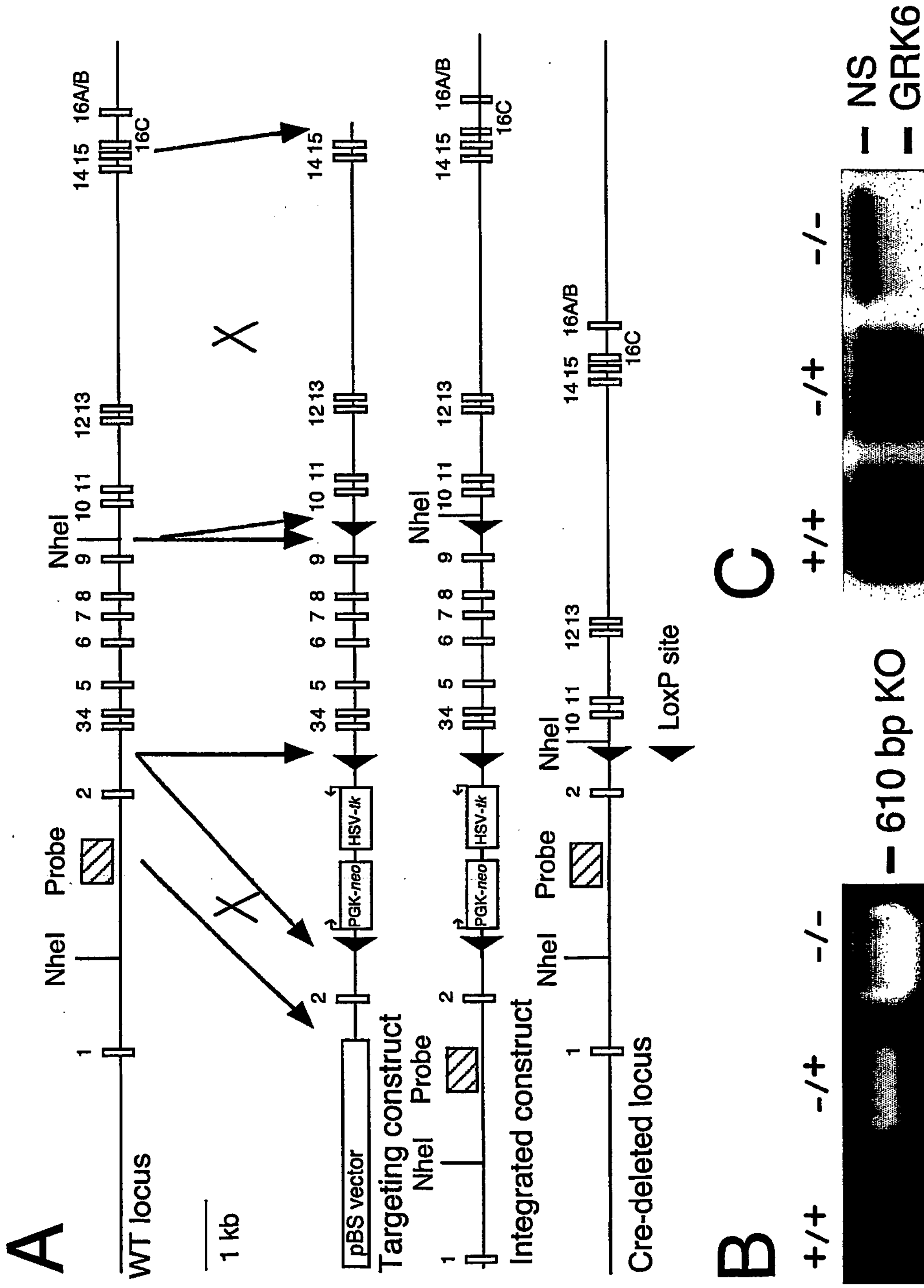

A
WT locus
1 kb
NheI
Probe
16A/B
16C
Targeting construct
pBS vector
PGK-neo
HSV-tk
Integrated construct
Cre-deleted locus
LoxP site
B
+/+
-/+
-/-
610 bp KO
460 bp WT
C
NS
GRK6

Fig 3

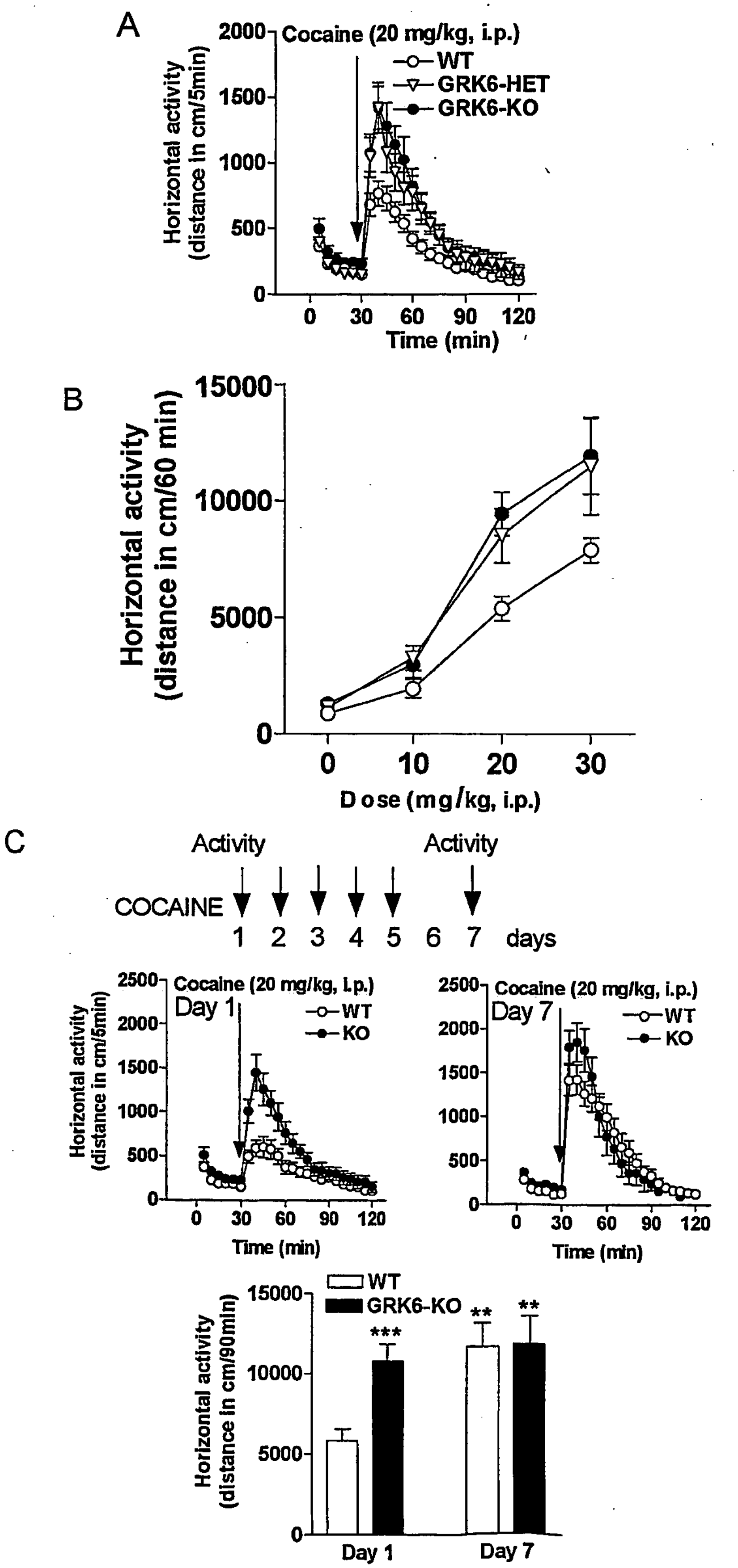

A
Cocaine (20 mg/kg, i.p.)
WT
GRK6-HET
GRK6-KO
Horizontal activity (distance in cm/5min)
Time (min)
B
Horizontal activity (distance in cm/60 min)
Dose (mg/kg, i.p.)
C
Activity
Activity
COCAINE
1 2 3 4 5 6 7 days
Cocaine (20 mg/kg, i.p.)
Day 1
WT
KO
Horizontal activity (distance in cm/5min)
Time (min)
Cocaine (20 mg/kg, i.p.)
Day 7
WT
KO
Time (min)
WT
GRK6-KO
***
**
**
Horizontal activity (distance in cm/90min)
Day 1
Day 7

Fig 4

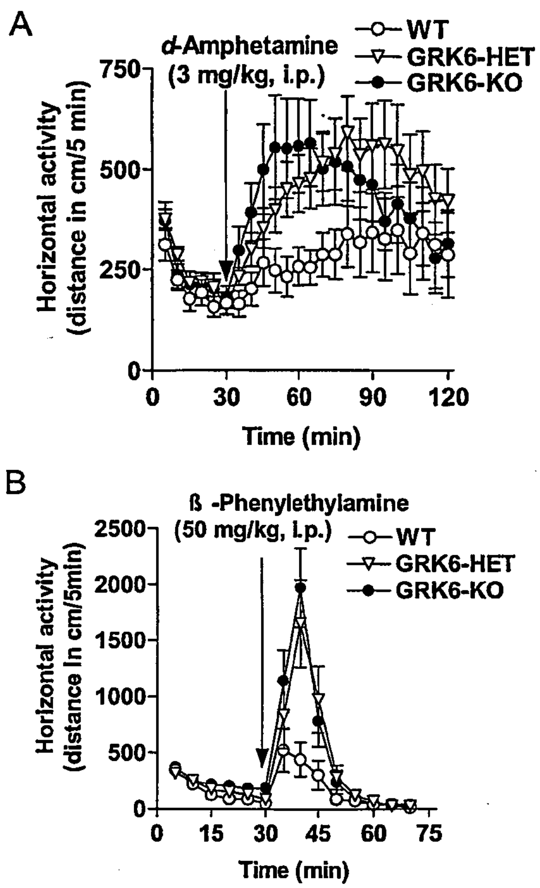

A
d-Amphetamine
(3 mg/kg, i.p.)
WT
GRK6-HET
GRK6-KO
Horizontal activity
(distance in cm/5 min)
750
500
250
0
0
30
60
90
120
Time (min)
B
ß -Phenylethylamine
(50 mg/kg, i.p.)
WT
GRK6-HET
GRK6-KO
Horizontal activity
(distance in cm/5min)
2500
2000
1500
1000
500
0
0
15
30
45
60
75
Time (min)

Fig. 5

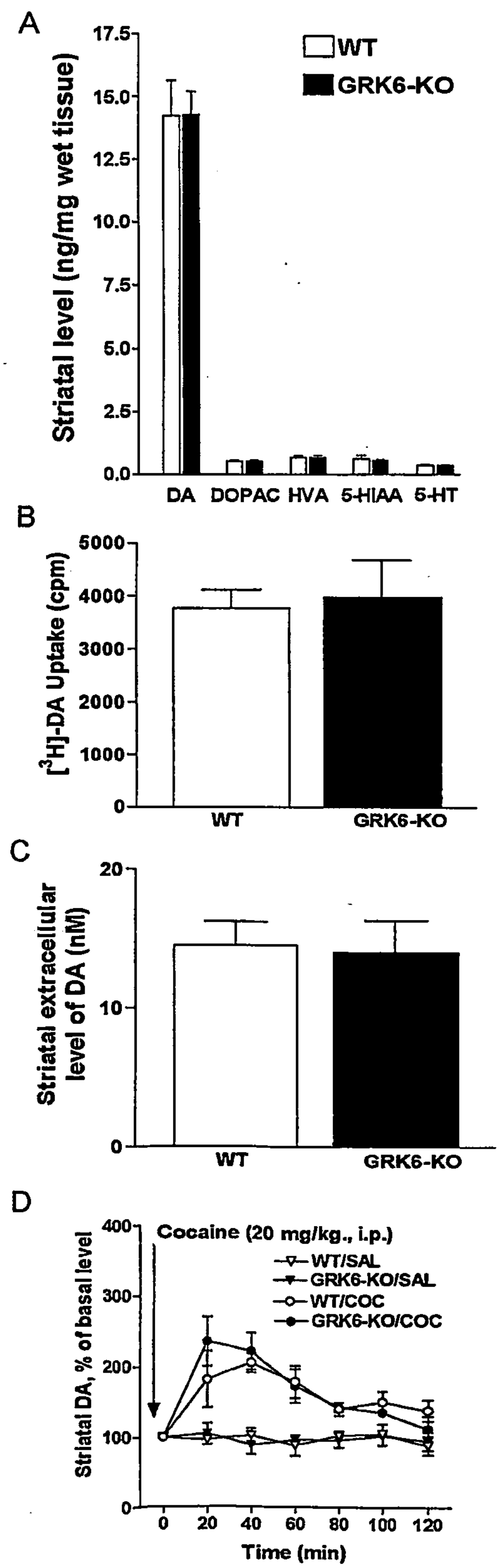

A
Striatal level (ng/mg wet tissue)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
WT
GRK6-KO
DA
DOPAC
HVA
5-HIAA
5-HT
B
[3H]-DA Uptake (cpm)
5000
4000
3000
2000
1000
0
WT
GRK6-KO
C
Striatal extracellular level of DA (nM)
20
10
0
WT
GRK6-KO
D
Striatal DA, % of basal level
400
300
200
100
0
Cocaine (20 mg/kg., i.p.)
WT/SAL
GRK6-KO/SAL
WT/COC
GRK6-KO/COC
0
20
40
60
80
100
120
Time (min)

Fig 6

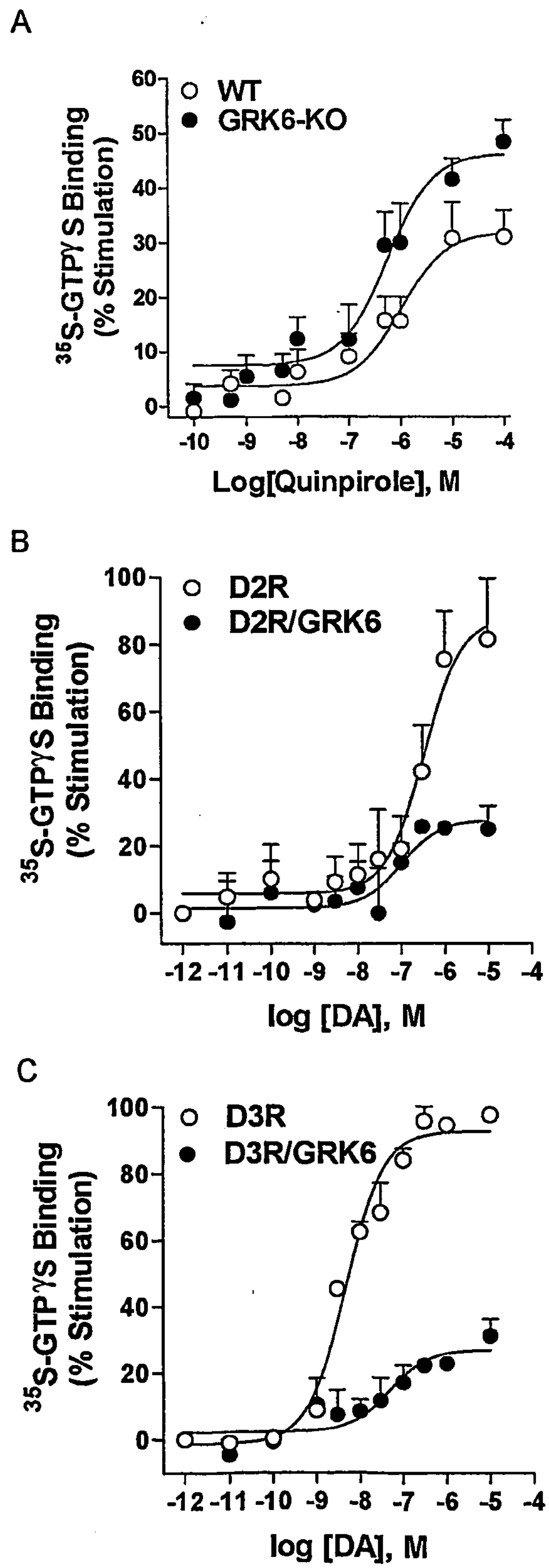

A
60
50
40
30
20
10
0
WT
GRK6-KO
35S-GTPγS Binding
(% Stimulation)
-10
-9
-8
-7
-6
-5
-4
Log[Quinpirole], M
B
100
80
60
40
20
0
D2R
D2R/GRK6
35S-GTPγS Binding
(% Stimulation)
-12 -11 -10 -9 -8 -7 -6 -5 -4
log [DA], M
C
100
80
60
40
20
0
D3R
D3R/GRK6
35S-GTPγS Binding
(% Stimulation)
-12 -11 -10 -9 -8 -7 -6 -5 -4
log [DA], M

Fig 7

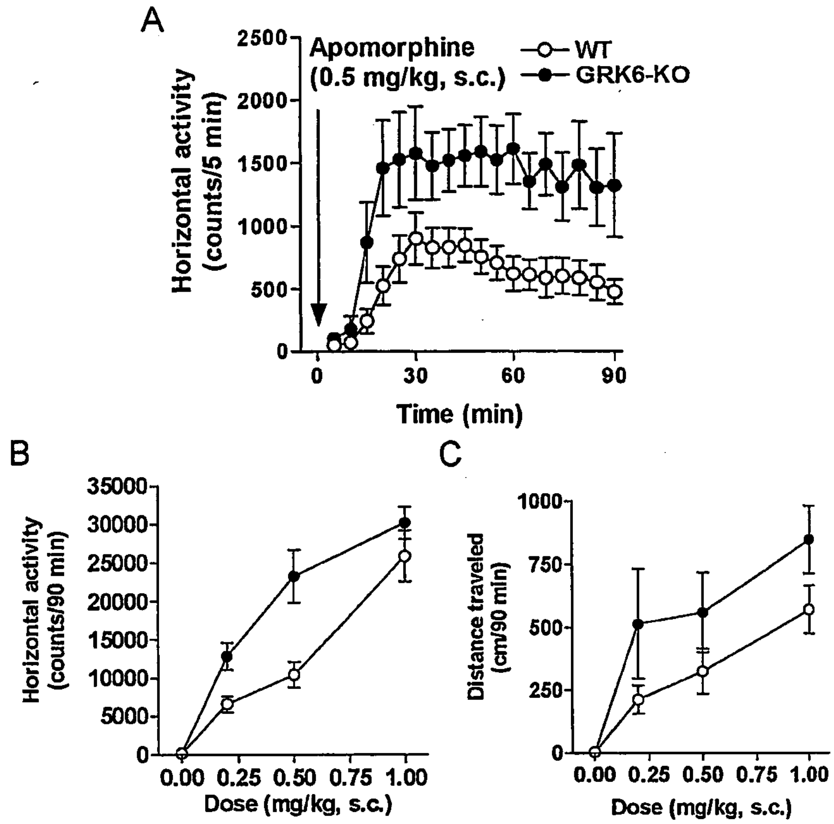
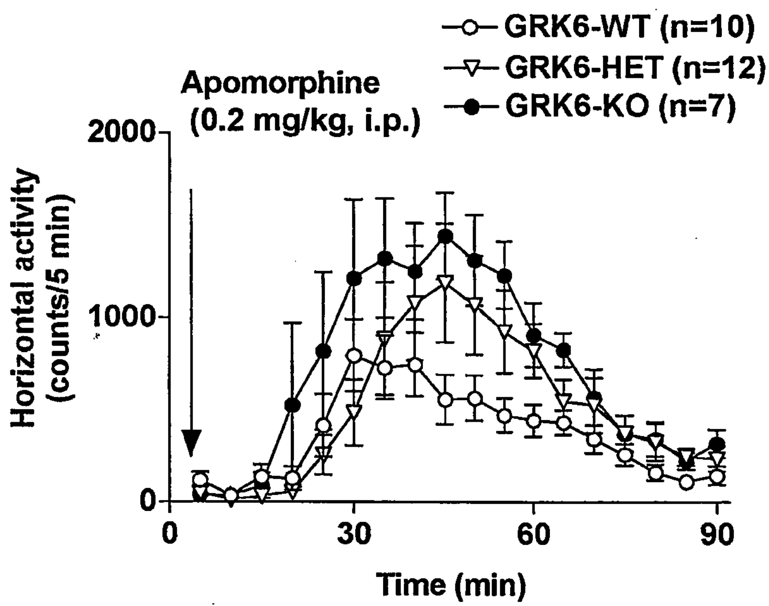

A
Apomorphine
(0.5 mg/kg, s.c.)
WT
GRK6-KO
Horizontal activity
(counts/5 min)
2500
2000
1500
1000
500
0
0
30
60
90
Time (min)
B
Horizontal activity
(counts/90 min)
35000
30000
25000
20000
15000
10000
5000
0
0.00 0.25 0.50 0.75 1.00
Dose (mg/kg, s.c.)
C
Distance traveled
(cm/90 min)
1000
750
500
250
0
0.00 0.25 0.50 0.75 1.00
Dose (mg/kg, s.c.)

Fig 8

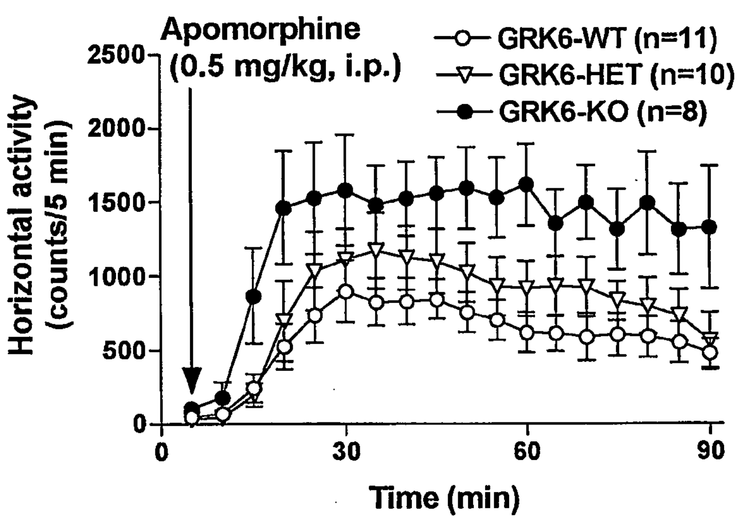
Effect of D1/D2 DA receptor agonist Apomorphine on the locomotion of DA-depleted GRK6 mutant mice



Mice were treated by reserpine (5 mg/kg, i.p., 24 h before) + AMT (250 mg/kg, i.p., 1 h before)

Fig 9

Effect of D1/D2 DA receptor agonist Apomorphine on the locomotion of DA-depleted GRK6 mutant mice



Mice were treated by reserpine (5 mg/kg, i.p., 24 h before) + AMT (250 mg/kg, i.p., 1 h before)

Fig 10A

GRK6/flox allele Sequence

```
         10        20         30        40        50         60        70         80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
TCTAGAACTGACTTGTAGACCAGGC TGGCCCCAAACTCAGAGATCCACCT GTGTTTTGAATGGCAAGTGCTACCA TGCCCAGCTTCTTGTCTTGTCTACT   100

TCTTTTTGTTTTTCTTCTTTTTTTT TTTTTTTCCAAAACATAATTCCTCT GTGTAGCCCTGGCTGTCCTGGAAGT TGCTCTGCCAACCAGGCTGACCTTG   200

AACTCAGATTTACCTGTGTCTGCCT CCCACGTGCTGGGATTAAAGGTGTG CCCCACCACTGCCCAGCTCCTATTC TCCTAACCTGTAGACTTCCCACTGT   300

GTTAGGAGTGAATGAGGCGGAACTT CTTGATGAGATGTCCTCATTGGTCA TTTTGGTTCTCATCCAGGGAACTCT TACCATGGGTGCCCACAAGGGCCAT   400

GTGTGTCCTGGAACTTGTTAAGGGC ATGCTGGAATGTTTGGAAGAAGCCT CAAGGTTCCTCCCGCAGCAGGTTTG GCCTTACTTAACAGGGCCCTGAAGG   500

CCTCTCTGTACAACATGTTTAGGGG AAGGTTCCGAGGCAGGCGGCTCAGG ACTCAATGGACCCAGTTCCTGATT GCTCTTGCAGGTGGTGGCGGGAATC   600
                                                                                          G  G  G  N  R
GCAAAGGCAAGAGCAAGAAATGGCG CCAGATGCTGCAGTTCCCCCATATC AGCCAGTGTGAGGAGCTTCGACTCA GCCTTGGTGAGGCCTGGCTCCCAGA   700
  K  G  K  S  K  K  W  R    Q  M  L  Q  F  P  H  I    S  Q  C  E  E  L  R  L  S    L  G
GCAGACTGGGGGGAGGGGAGCTGGC GGGGGTAGCCATGAGGAGTCATCCC CAGACATATCCTTGGCCATGGGGGC CTGGGGGGGGGGGAATTCAGGAAGA   800

CCGAGACGCCATAGTCCAGTTTCAG TTCCTGGGCCTGAAATGGCAGAGGG CAGAGAACGGAGACTGGTGTCAGCA GAGTGGGCATGGGCGAAGGCAGAGG   900

GCCAGGATGGAGGGAGGTGAGAGAA ACTTAGGAGGGAAGTCAGGTTTGGA ACACCAGTCCTGCGCTCATGAGCTT TGCACCTGAGGGTACCCATCAAAGC  1000

TAGGCAGTATGGTGGTTACTTCTA GAAGGGCTCATCCCAGTTCCAGCAT CGTGGATGAGGGTCTGAACTGTATG TACCTCAGGTGGCCCCGTCCTCTGC  1100

TGATGGCTTGCATGCGCTTGGGACG GAGGCCCAGCTGAGAGCAGCGGAAG CCACAGCTTCCTCTCGCACTTTCGC CAGGCAGGCAACAGGTGTAGACTTG  1200

GGCTGGGCTGGGCGGCAACCACACA GACCACACCCAACTCCAGAACTGTG ACAAGCAAGTCCAGGAAACAACAGG GAAAGGAGACCTGGTGTCTAGAGGC  1300

GCGCCATAACTTCGTATAGCATACA TTATACGAAGTTATCCTGCAGGGTG CTCGAGCACACTAGAGCCCAGACTC CTGGCTGTGTGGCACTGAGTTGAAT  1400

TAATCAACCCCTCTGGCCCCATAG CCCCCAGGTTACATTGGACTTGCAG TGGCGCCCACCTGAAGATTGCAGGG AGGGTTCTGAAGAGATAATGACTGC  1500

CACTTTTTGGGGCTAGAAGTTATTG GATTCCAGATGTGGGATATAGAAC AGAGTGCCCTGCCAGTGAGTTCTAG GTCTGTGACCTCGCAGTTCCCCTCT  1600

TGGCCTGCAGAGCGTGACTACCACA GCCTATGTGAGCGCCAGCCCATTGG GCGCCTGTTATTTCGTGAGTTCTGT GCTACGAGACCTGAGCTGACCCGGT  1700
            R  D  Y  H  S   L  C  E  R  Q  P  I  G   R  L  L  F  R  E  F  C   A  T  R  P  E  L  T  R  C
GTACTGCCTTCCTGGATGGGGTGGT GAGTATCCCACCCAGGGCCCAGCCC AGCACTGAGGGCAGGCAGAGGTCCT GGCCATTTTCCTCCCCCCCCCCCCC  1800
  T  A  F  L  D  G  V
CGCCCTAATCCGTCCCCACATCTGT CAGGTTCTTTGCCTCTCACCCTGCA GTCTGAATATGAGGTGACCCCTGAT GAGAAGCAGAAAGCATGTGGCGCC  1900
                                                     S  E  Y  E  V  T  P  D   E  K  Q  K  A  C  G  R  R
GACTAATGCAGAACTTTCTGAGCCA CACGGTGAGTGAGCGGTGACAGGGA GATGACAGCAGCAGGCAGGGCCCAG TGACAACAGCACAGGAGTGACCACA  2000
  L  M  Q  N  F  L  S  H   T
GCCTGGGCACTGAGTGCCCGGGAGC TGTTCCAGGCAGCCCCAAGGGCATG GAGCCCAAGGGTTGGGCTGAGCTTG GGTCAGGCAGCCTGCCAGGGGCTGG  2100

CTCACCCTGCCTTAAGGGCCGGCAG AAAGGGAGTTGAACAAACTGGCAGC TAGTGTGCACTTGCTACCCATAGCC GCTCTCGGCACCCACAGACATCTGC  2200

CTGTGCTGCCCCGGGAGTAGGCAGT TGTCCAAGGGAGCCCTCTTGCATTA GGTGTCTGTGGCATGTCCCTAGGGT CCTGACCTCATCCCTGAAGTTCCAC  2300
                                                                          G   P  D  L  I  P  E  V  P  R
GGCAGCTGGTGAGTAACTGTGCCCA GCGGCTAGAGCAGGGACCCTGCAAA GACCTCTTCCAGGAGCTGACCCGGT AAGGCTCCATACCTCCTGGTCTGGA  2400
  Q  L  V  S  N  C  A  Q    R  L  E  Q  G  P  C  K    D  L  F  Q  E  L  T  R
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CCTGCTTCAGTGTAGAGTGCATGCA CCCTCCTGCTCAGGGCCTCAGTGAG AAGCTTGCCGAAGGAAAAGGATGTT GCTTTGTGTCTGACCGGCATGTAGT  2600

GGGAGGCCTGTGCCTTCAGGCTGAG AATTGGCCTTGTTAGAGGCTCGCCT ACAGACTGATCCTCTCTCAACAGGC TGACCCACGAGTACCTGAGCACGGC  2700
                                                                              L  T  H  E  Y  L  S  T  A
CCCTTTTGCCGACTACCTCGACAGC ATCTACTTCAACCGTTTTCTGCAGT GGAAGTGGCTGGAAAGGTGAACGCC TCCCAAGCTGGCCTGTGGTAGGTCA  2800
 P  F  A  D  Y  L  D  S   I  Y  F  N  R  F  L  Q  W   K  W  L  E  R
GATTGTGGGCTCTCATGGCAAGCCC TGCCTGTGTAAATGTTTGTTTTTTT ACTCTCGCCTATAGGCAACCAGTGA CCAAAAACACCTTCAGGCAGTACCG  2900
                                                                    Q  P  V  T   K  N  T  F  R  Q  Y  R
```

Fig 10B

GRK6/flox allele Sequence

```
       10        20         30        40        50         60        70         80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
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 V  L  G  K  G  G  F  G   E
GCAAGAGCATGGCTGGCCCCCATCC ATCCCTGTCTGCCTGGTTACAGGTA TGTGCCTGCCAGGTGCGGGCAACAG GCAAGATGTACGCATGCAAGAAACT   3100
                                                 V  C  A  C  Q  V  R  A  T  G   K  M  Y  A  C  K  K  L
GGAAAAGAAGCGGATAAAGAAGCGA AAGGGGGAGGCCATGGCTCTCAACG AGAAACAGATCTTGGAGAAAGTGAA CAGTAGGTTTGTAGTAAGTACACAA   3200
 E  K  K  R  I  K  K  R   K  G  E  A  M  A  L  N  E   K  Q  I  L  E  K  V  N   S  R  F  V
GGAGCCCTCTCCCTTCCCCTGGGCC ACACCACCTGCTACATTCCCCACCC ACCAGGCTAAATTCCCTCCCTATTG CCAAAGGGACTGCCCTCTGCCCCCT   3300

CCCTTCCTGCCCTGGACAGTACCCA AGAAGGGTGGGGTAGGAGTGCTATT CCAGGCTCAGGAAGCCTGCTGGGTC CTAAGGAGTGGCACACAGAAGATGG   3400

TGGCTGCCATTAGCATTTAGGAGAG TGAGCATGCGTGCGAACAGGTTCCT GGCTGAGGGTGTGGGTCTCCCGAGC ACAGGTGAGCTTAGCCTACGCCTAT   3500
                                                                                 V  S  L  A  Y  A  Y
GAGACCAAGGATGCACTGTGCCTGG TGCTGACATTGATGAATGGAGGTGA CCTAAAGTTCCACATCTACCACATG GGCCAGGCTGGCTTTCCTGAAGCAC   3600
E  T  K  D  A  L  C  L  V   L  T  L  M  N  G  G  D   L  K  F  H  I  Y  H  M   G  Q  A  G  F  P  E  A  R
GTGCTGTCTTCTATGCTGCTGAGAT CTGCTGTGGCCTGGAAGACCTGCAC CGGGAACCCATTGTGTACAGCTAGG CCCGGGCTCGGGCGGCTGGCTGTTC   3700
 A  V  F  Y  A  A  E  I   C  C  G  L  E  D  L  H   R  E  R  I  V  Y  R
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AACAAGTCCAAGGATGACTTCCAGG GACCCCTCTCCCCAGGTGCTCCCCC ACTGGGTTGCAGTCCTCAGTTCTTA CTACTCTAGAAAGTTCCTGAGGTCC   4300

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AAAACAGGCCTCTGACCCTGTGTTT CTCTCCCCATAGGGATCTAAAGCCA GAGAATATCCTTCTGGATGACCATG GTGGGTGAGAAGGCCCAAGTGGGAG   4500
                                        D  L  K  P   E  N  I  L  L  D  D  H  G
ATGGGGTGAGGTTGGAAGCTAGGCC TATCAGTGAACCCACCTGCTTCCAT ACCCCTACCCAGGCCACATTCGGAT CTCGGACCTGGGACTGGCCGTGCAT   4600
                                                                     H  I  R  I   S  D  L  G  L  A  V  H
GTGCCTGAGGGCCAGACCATCAAAG GCCGTGTGGGCACTGTGGGCTACAT GGGTAAGTCCTGTTGACCTAGTACG CCAGTCTCCTCAGCCCCCTTGCTGT   4700
V  P  E  G  Q  T  I  K  G   R  V  G  T  V  G  Y  M   G
CCCAGGCCTGGCCTACTTACTCACT GTTGTACTAGGGGAGGGAGGGTGCT AACTTCAGAGAGTAGGTGTAGGTGT GTGTGTGTGTGTGTGTGTGTGTGTG   4800

TGTGTGTGTGTGTGATGTGGGGAAA CCAAGACTTAGCCAGATCCCACCAT TGTGAACCCTGCTATTCATTTGCCG GATGCTTATTGAACACCTAGGACTG   4900

GGTGGGCCTGTGAGGGTGAAGGGAA ACAGAAACAGAGTACACATCCATGC CCCATGCTTGGATAGCATGCCATTC ACAGGGGACACAGGCAGTGGAGACC   5000

TAGCTTGTGGACAGTGTGACACAGT ACAGTCTAGGCTCTCCAGAGTCCAT CTTGGGCTAGGCATTCCACTTCCTG CCTTCTTTCTCCTACTGTAAAACCT   5100

CATCAGAGCTGCCTCCTGGGGGGCT TTGAGGTGAAAGGAGATGCTAAATG GAGAACTGTGCATGGGGCCTGCCAG GGATGGTGGAGGTAGCAGCCAGTG    5200

GGCGTTTAAAATCGCTTGTGAAAGC CAAAAGTTACAAATCAGTAGCTACG TGGTCCTGATCTGGCATACATTAAA TTTGAAGTTGAATTGTTTTTAGAAT   5300

AATTTGAATTGATTGCCAGCATTTA AGGCAAACAACTTAATGTATTAAAA AAAAAAAAAAAAAAAGGTGTATGGG GTCTTGGTTGGGTGTGTATGATGTA   5400

ACTCAGAGTACGTGCCTGATAGTTG TGTGGCCCTGGGTTCAACAAACTCC AGCACTAGGAATCCCACTCCTGCCC CCAGGGAAGACAAGCAATCAGACAC   5500

GGAGTTCCCTTGGTGTGACCCTCCC CTCAGGGCCACACCAGCCACCATTG ATGAGATGTGGCTTCTCATACTGGC TCAGTCCACAGCAGGCCAGTGGCAT   5600

ACCTGCCTATCCAGAGGATGTTTGA TCAAACTCTGGTTTTTGTTTCTGGG GCCCGAGGGCTCCCTGCTCCTCACG ACCTGCCCGGTCCTGACTCCTGGTC   5700

CCTGTCCAGCTCCAGAGGTGGTGAG GAATGAGCGCTACACGTTCAGTCCT GACTGGTGGGCGCTAGGCTGCCTCC TGTACGAGATGATCGCAGGTCAGTC   5800
         P  E  V  V  R   N  E  R  Y  T  F  S  P   D  W  W  A  L  G  C  L  L   Y  E  M  I  A  G  Q  S
```

Fig 10C

GRK6/flox allele Sequence

```
        10        20         30        40        50         60        70         80        90        100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
GCCCTTCCAGCAGAGGAAGAAGAAG ATAAAGCGCGAAGAGGTGGAGCGGC TGGTCAAGGAAGTGGCCGAGGAGTA CACAGACCGCTTTTCCTCACAGGCG  5900
 P  F  Q  Q  R  K  K  K   I  K  R  E  E  V  E  R  L   V  K  E  V  A  E  E  Y   T  D  R  F  S  S  Q  A
CGCTCACTCTGTTCTCAGGTACAAG CCAGAGTCTTAGCTGGGGGCAGCTG GGGTACCTCACCCACTCAGGTACCC TCACCACCACTGCCCTTCTATAGCT  6000
R  S  L  C  S  Q                                                                                    L
TCTTAGCAAGGACCCTGCTGAGCGC CTGGGGTGTCGTGGAGGTGGCGCCC GTGAGGTAAAGGAGCACCCCCTTTT CAAGAAACTGAATTTCAAGCGGCTG  6100
 L  S  K  D  P  A  E  R   L  G  C  R  G  G  G  A  R   E  V  K  E  H  P  L  F   K  K  L  N  F  K  R  L
GGAGCTGGCATGCTAGAGCCACCTT TTAAGCCTGATGTAAGTCCTGCCCT CCCTTGCTAGTGGCCAGCCGAGCCA GGGGGTGGGGCGGGGCAGGGCTGGG  6200
G  A  G  M  L  E  P  P  F   K  P  D
GTGGGTGACTGGGAGCAAGGGCCAA GTCACTTTCAGTGCAGATGTTGGAA AAACCCCATCTAATGGCTTGGGCTG CCCCTCACCCCCAGTTTGTGTGTGT  6300

GTGTGTGTGTGTGTGTGTGTGTGTG TGTGTGTGTGTGTGTGTAGGCTTGT GAAAATGGAATGCCCAACATACTGT GGGTTTTAGTGTACCAGGTTAGAAT  6400

GCTGGGCAGACATGGAGATGCTCTG GGAGCATTGCAAGAACTCATGTCCT CTCTGTACTGTGATTGGTTAGCCTT CGATGGCAGGCATCTTTTCCCAGGG  6500

AGTGGCAATGGTGACCTAGTGGCTC AGGCTCATGTCTTCTTAGATAGCCA CCCAGTGGGCACAGAGCAAGGTTTG TTCACGAATTTGAAATTAAATGCTC  6600

TGTTTGCTGAAGGGCCCCACTGGAC ACCCAGATCACCCCTTCCTGGCCCA ATGGGAATAACCCATTTCCAAAAGG AACTGAGACGGTGGTGCCAGCAGGG  6700

GCTGTTGGGCTAACTGCCCCTCTGC AGGCAGGCAATTACCCCCTTCAAAG TTTAACTTTAAAATATTTATCTTAT TCTTAATTTTGTGTTTGTGTGTCTG  6800

TCTCTGTGTGGGTATGTATATGTCT ATGTATGTGTGTATGTCTAGGAATG TGAATATGTCTATGTATGTGTGTGC CTGTGGAGCATGTGTATGTCTGTAT  6900

GTGTGCGTCTGCGTGGGACGTGGA TAGGTGTGCAGTTGCCACGGAGGCC CGAAGCTTTAGATCCTCCTGGGAAT GGAGTCATGGGTGGTTGTGAGTGTC  7000

TGCATGACATTAGTTTGAGGAATCA ACTTAGATCCCCCACAGCAGCAGTG CATACTTCTAACTGCTGAGCCCGTC TTTCTAGTCCCAGAATCTCCCTCTT  7100

GAGAGTTTTACCTCTGAGCTAAGCA TCTTGTGGATTGTACACCGTGCAGA CAATTGTACTCTAGATATTAGAGCC TAATAGCAGAAACCCTGCTCAGACT  7200

CACGTCAAGTCTGTTGGTTCATTTG TCCCCAAGTCTCTCTGGGCCATCAC TGGGGCTTTGTGCTGGGTTGCTAAG ACTGTCTCTTGGCTCTTCAACTTGT  7300

CTGTACAGAACCTGAATTCAGGTTT CTCTGTAATGTCAGAGGAAGGGTTC TGGTTGGCCAGGATGGGGTCATGTC CTTGCCTCCCTCCCTTAAGTTTGTG  7400

GACACGGGGGTCCTGTGATTTGACA GTTTGCCACTTGGGAAAGTCTTGAG GAGATGGGGGCACCAGGTTGATGGC TGTCAGCCACATATGTTTATGCGTC  7500

TGACAAATACCTGAAAATCTGTATG AGTCAGGTCTTTGAGATAATGAGGT TCATAGAGCCAGGCCTATTCTCATA CTATAGTGGAGAGAAATAATGAAAA  7600

TAAACTAACAAGCAAAGAGTGTTTT CAGATAGTCCCAAGTCTGACAGGAA AAAGAGCAGGCAAAGAAGGCAGTGA TGTGGGTGTGGTCGTCTCTGAGAGA  7700

GTGATGTTTACGCAGAACTGAAGGA GAGAGTCTCGCAAGATGAGAGGCCA CAGGCATTAGAGGCAGAAGGGACAG GCAGGCACTGAGGGCCAGTGGCAGG  7800

GTCAGCCTAGGGACATGGAACCACC ACCATGAGGCCATCGTGTCCGGAGC AAGTCAGTGCAGAGCAGCAACAGTG AATGTCGCGATGTTCTAATGATGAC  7900

AGGAGACCTTCGGGCTGCAGCTCTA ATGTGAGGTGTGGAAATGGGTCATA GAGAATGAGTGAGCTGGGGAAAAGT CAGCGAGGGGGCCTCTGAGTGATGG  8000

GTCACACAAGTGAGGGTAGTGCCTC TGAGTGAGGACTCACACAGTGGGGG AGGCGTTCTGAGTGATGGCTCCCAT GCTGCAGGTGAGCACTGTCAATGAG  8100

TTGGCACTTGAACCGGACAGGTCTC ACTTCATCCTGCTGCTCCAGGCTGG AGCTACTGCACAGGTTGACTCCTCT CTCTGTACATCATCTAAGTAATTGC  8200

ACGGCCCGTGGGTCATCTGTTCAAA GTCAGAAAATAAGGACAGTGTCTTG GGACTTAGAAGTAGCAGATGAAAAA ACAAATTTAACAATGCTTTTATGGT  8300

TTTAATTTAAAAAAAAAAATTACAT GTCTACCTATGTATCTGTGTGTGAG TGTGTGCATGTGAATTTAGGGGCCC CTGGAAGAACAGTGCAAGTTTTTAA  8400

CCTCTAGACCTGCACTTGACAATAT TGGGGGCGGGAGGGCGGGTTTCTCT GTGTAGCCCTGGCTGCTCTGGGACT CACTCTGTAGACCAGGCTGGCCTTG  8500

ACTCATAGATCCACCTGCCTCTGCC TTCTGAGTGCTGGGACCAAAGGTGT GCACTACCATGCCCAGCCGACATAC ATTTTTAAAAAAGATTTATTTTATG  8600

TATATGAGTACATTGTCACTGTCTT CAGACACACTGGAAGAGTGCATCAG ATCTCATTATAGATGGTTGTGAGCC ACCATGTGGTTGCTGGGAATTGAAC  8700
```

Fig 10D

GRK6/flox allele Sequence

```
         10        20         30        40        50          60        70         80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
TCCGGATCTCCAGAAAAGCAGTCAG TGCTCTTAACTACTGAACCACCTCT CCAGCCCAACAACACATTTTGAACA ACTCTGTTGAGATGAAACTCACAGT  8800

CAATCCATATAAAGTATTCATGTCA ACTGAAAGAGTCACGTGACTGTCAC CACAAAAGTTCAGAATGTTTTTGCC AGCCCTTTCAGAACATAAGCAAAAC  8900

CTTATGTCCATTAGGGTTCCAAATC CTTTCCTCCTACCCCAAGAAACCAC AGCTGTCTGTCTTCAACTAGTTTAG ATGCTTCAGTTGTGTGGTCAGATGT  9000

GGGCATGCTGAACACGGTCCTCTGT GACTGGCTTCTTTTGCTAAGCATGG TTTTGTTTTGTTTTGTTTTGTTTTT AAGTTTATCAAGACAGGTTTTCTCT  9100

GTATAGTTCTGGCCGTCCTGGAACT CACTCTGTAGACCAGGCTGGCCTCC AACTCAGAAATCCACCTGCCTCTGC CTCGCTAAGCATGCTTTTAAAGTTC  9200

ATCTATGTCGGAGCATTCTTCTTAA AAGCTGGTTAATATTCTGTAACGTG GATATAAAATAAACCTTATTCGTTC CTGGTTGACATGAGGGTGTTTATCT  9300

TTTTGAGGGGTGGGTCTATCAGGAA TAATCCTGTCGGCTTTTTTGAGATG AGGTGCCATTGCATTGTCTTGTCCA GTTTTGAAATGGCAGACTCGGGCAT  9400

TCTTCCTGACCTCAGTATATACATT GACGGAACATTATGAAGTCAAAAAT ACTGTATCTTGTAGGGAGCTTTAGA CCTTTCCAGGTTAGGTACACCATTT  9500

TACATGATGGTGATGATCATGTATG TATCAGGCAGGTTGGTAGGTATGTG TGTGTGTGTGAGAGAGTGTGTGTGT GTGTGTGTGTGGTCTATGTGTGTGA  9600

GTGTGTGGTCTATGTGTGTGAGTGT GTGTGTGTGAGTGTGTGTGTGTGTG TGTGTGTATTGAACCCAGGGCCTAA AGCATACTGAACCACTGTTCTATCA  9700

CTGAACTACACCTCTGACTTCATGA TGGTAATATGTTAATTGACGTCCTT TGTGGTAAGATTTTGAAAGCAGCAC AGATTGAAAATAAGAAAGCTTCAAA  9800

TACAGAAGCCAGTTCCCAGCAGGAA CTGGGAGGGCGGCTGGTAAGACCAA TCCAGTACCGGACATCCCAGAGTTG AGACACGCACACACACACACACAGT  9900

CCCTATGGAGGGAGCCAGGACTCCC CTTCCCACCCCAACCCCAACACATA CCCAGGCAGTCCTGTGCAAGTACAA GGTCAGCCTAACACCAGGTCTGCAC 10000

ACAGCCTGTCCAGGCTCCGCACAGC GAGGGTCACGATCCTCGTCTATGGA GGCGAAAACTGCCTCAGAGACAGTA GAGTGCTGAGCTGAGGAGTGTGTGG 10100

GGCACCATGCTGATCTCTCCAAATC AGGATGCTGCCAAGGGTGGCAGACT CAAGCCCTCCTGACTCCTGCCTCTT CCACCTTAGCCCCAGGCTATTTATT 10200
                                                                                       P  Q  A  I  Y  C
GCAAGGATGTCCTGGACATTGAACA GTTCTCTACAGTTAAAGGTGTGGAT CTGGAGCCCACAGACCAAGACTTCT ACCAGAAGTTTGCCACAGGCAGTGT 10300
 K  D  V  L  D  I  E  Q   F  S  T  V  K  G  V  D   L  E  P  T  D  Q  D  F  Y   Q  K  F  A  T  G  S  V
GTCCATCCCCTGGCAGAATGAGGTA GGGATTATCCTGTGACTGGGATGAG GTCCAGGTAGGGCACCCCCAGGGCC AACTCTCACCGCTTCCTGTTCCTGG 10400
 S  I  P  W  Q  N  E
GTAGATGGTGGAGACCGAGTGCTTC CAGGAACTCAATGTCTTTGGGCTGG ATGGGTCTGTTCCCCCAGACCTGGA CTGGAAGGGCCAGCCCACTGCACCC 10500
   N  V  E  T  E  C  F   Q  E  L  N  V  F  G  L  D   G  S  V  P  P  D  L  D   W  K  G  Q  P  T  A  P
CCCAAGAAGGGATTGCTACAGAGAC TCTTCAGTCGCCAAGTAAGTCCTAG CAGTGTCTGCCCTGGGTCCCACCTG CTCCACTGGGAGAGTGGGTGGGAGG 10600
P  K  K  G  L  L  Q  R  L   F  S  R  Q
CAGAGCTGGTGCATGCCTGCCCCGG GAGTGGGCATCTTCCTGTGGTGCCC AAGGTGCCTCATTCCTGCCTGTCCC CCCTGACCCCCACGCCTGGCTGGTC 10700

TCATGGCCTCTTTTCTCTTTCCAGA GGTGAGCAGTGTGGGCTCCCCATGG GTTGGCCTTGCTGCCTGACTGTGGG GAGCCCCAGGCAGCCCATCCGAGGC 10800
                         R  .
AGAGGCGAGATGTAGGAGAAAGAAG TCTGGTGTCCACCTTACCCGCTCCT TCCCTGCCGCTTGCCTCCCTGCACC CTGACCCCTTCAAGCTGCTAACCTT 10900

GCTTAGCAACTGTCTCCTTGTGCCA GAGTCTGTCCAGGGGGATGGACAGA GGCCTTGCTCTGACAGATCGAGTCG ACGCCCTATAGTGAGTCGTATTAGA 11000

GCTCGCGGCCGC                                                                                                  11012
```

Fig.10E

GRK6/flox construct Feature Table

Key	From	To	Strand	Shown...	Description
misc_feat...	1	6	5'	Boxed/N...	Xbal site
exon	586	681	5'	AA1/Fat...	exon 2 /codon_start...
misc_feat...	1292	1297	5'	Boxed/N...	Xbal site
misc_feat...	1339	1306	3'	Underli...	loxP site
misc_feat...	1360	1365	5'	Boxed/N...	Spel/Xbal junction
exon	1611	1723	5'	AA1/Fat...	exon 3 /codon_start...
exon	1852	1929	5'	AA1/Fat...	exon 4 /codon_start...
exon	2273	2373	5'	AA1/Fat...	exon 5 /codon_start...
exon	2674	2766	5'	AA1/Fat...	exon 6 /codon_start...
exon	2865	2928	5'	AA1/Fat...	exon 7 /codon_start...
exon	3048	3188	5'	AA1/Fat...	exon 8 /codon_start...
exon	3480	3670	5'	AA1/Fat...	exon 9 /codon_start...
misc_feat...	4097	4102	5'	Boxed/N...	Nhel/Spel junction
misc_feat...	4142	4109	3'	Underli...	loxP site
misc_feat...	4143	4148	5'	Boxed/N...	Nhel site
exon	4438	4475	5'	AA1/Fat...	exon 10 /codon_star...
exon	4563	4652	5'	AA1/Fat...	exon 11 /codon_star...
exon	5710	5918	5'	AA1/Fat...	exon 12 /codon_star...
exon	5999	6136	5'	AA1/Fat...	exon 13 /codon_star...
exon	10185	10322	5'	AA1/Fat...	exon 14 /codon_star...
exon	10405	10539	5'	AA1/Fat...	exon 15 /codon_star...
exon	10725	10730	5'	AA1/Fat...	exon 16-C /codon_st...
misc_feat...	11012	11005	3'	Boxed/B...	Notl site (from gen...

Fig 10F

SEQ ID No: 1
TCTAGAACTG ACTTGTAGAC CAGGCTGGCC CCAAACTCAG AGATCCACCT 50
GTGTTTTGAA TGGCAAGTGC TACCATGCCC AGCTTCTTGT CTTGTCTACT 100
TCTTTTTGTT TTTCTTCTTT TTTTTTTTTT TTCCAAAACA TAATTCCTCT 150
GTGTAGCCCT GGCTGTCCTG GAAGTTGCTC TGCCAACCAG GCTGACCTTG 200
AACTCAGATT TACCTGTGTC TGCCTCCCAC GTGCTGGGAT TAAAGGTGTG 250
CCCCACCACT GCCCAGCTCC TATTCTCCTA ACCTGTAGAC TTCCCACTGT 300
GTTAGGAGTG AATGAGGCGG AACTTCTTGA TGAGATGTCC TCATTGGTCA 350
TTTTGGTTCT CATCCAGGGA ACTCTTACCA TGGGTGCCCA CAAGGGCCAT 400
GTGTGTCCTG GAACTTGTTA AGGGCATGCT GGAATGTTTG GAAGAAGCCT 450
CAAGGTTCCT CCCGCAGCAG GTTTGGCCTT ACTTAACAGG GCCCTGAAGG 500
CCTCTCTGTA CAACATGTTT AGGGGAAGGT TCCGAGGCAG GCGGCTCAGG 550
ACTCAATGGG ACCCAGTTCC TGATTGCTCT TGCAGGTGGT GGCGGGAATC 600
GCAAAGGCAA GAGCAAGAAA TGGCGCCAGA TGCTGCAGTT CCCCCATATC 650
AGCCAGTGTG AGGAGCTTCG ACTCAGCCTT GGTGAGGCCT GGCTCCCAGA 700
GCAGACTGGG GGGAGGGGAG CTGGGGGGGG TAGCCATGAG GAGTCATCCC 750
CAGACATATC CTTGGCCATG GGGGCCTGGG GGGGGGGGAA TTCAGGAAGA 800
CCGAGACGCC ATAGTCCAGT TTCAGTTCCT GGGCCTGAAA TGGCAGAGGG 850
CAGAGAACGG AGACTGGTGT CAGCAGAGTG GGCATGGGCG AAGGCAGAGG 900
GCCAGGATGG AGGGAGGTGA GAGAAACTTA GGAGGGAAGT CAGGTTTGGA 950
ACACCAGTCC TGCGCTCATG AGCTTTGCAC CTGAGGGTAC CCATCAAAGC 1000
TAGGGCAGTA TGGTGGTTAC TTCTAGAAGG GCTCATCCCA GTTCCAGCAT 1050
CGTGGATGAG GGTCTGAACT GTATGTACCT CAGGTGGCCC CGTCCTCTGC 1100
TGATGGCTTG CATGCGCTTG GGACGGAGGC CCAGCTGAGA GCAGCGGAAG 1150
CCACAGCTTC CTCTCGCACT TTCGCCAGGC AGGCAACAGG TGTAGACTTG 1200
GGCTGGGCTG GGCGGCAACC ACACAGACCA CACCCAACTC CAGAACTGTG 1250
ACAAGCAAGT CCAGGAAACA ACAGGGAAAG GAGACCTGGT GTCTAGAGGC 1300
GCGCCATAAC TTCGTATAGC ATACATTATA CGAAGTTATC CTGCAGGGTG 1350
CTCGAGCACA CTAGAGCCCA GACTCCTGGC TGTGTGGCAC TGAGTTGAAT 1400
TAATCAACCC CTCTGGGCCC CATAGCCCCC AGGTTACATT GGACTTGCAG 1450
TGGCGCCCAC CTGAAGATTG CAGGGAGGGT TCTGAAGAGA TAATGACTGC 1500
CACTTTTTGG GGCTAGAAGT TATTGGATTC CAGATGTGGG GATATAGAAC 1550
AGAGTGCCCT GCCAGTGAGT TCTAGGTCTG TGACCTCGCA GTTCCCCTCT 1600
TGGCCTGCAG AGCGTGACTA CCACAGCCTA TGTGAGCGCC AGCCCATTGG 1650
GCGCCTGTTA TTTCGTGAGT TCTGTGCTAC GAGACCTGAG CTGACCCGGT 1700
GTACTGCCTT CCTGGATGGG GTGGTGAGTA TCCCACCCAG GGCCCAGCCC 1750
AGCACTGAGG GCAGGCAGAG GTCCTGGCCA TTTTCCTCCC CCCCCCCCCC 1800
CGCCCTAATC CGTCCCCACA TCTGTCAGGT TCTTTGCCTC TCACCCTGCA 1850
GTCTGAATAT GAGGTGACCC CTGATGAGAA GCAGAAAGCA TGTGGGCGCC 1900
GACTAATGCA GAACTTTCTG AGCCACACGG TGAGTGAGCG GTGACAGGGA 1950
GATGACAGCA GCAGGCAGGG CCCAGTGACA ACAGCACAGG AGTGACCACA 2000
GCCTGGGCAC TGAGTGCCCG GGAGCTGTTC CAGGCAGCCC CAAGGGCATG 2050
GAGCCCAAGG GTTGGGCTGA GCTTGGGTCA GGCAGCCTGC CAGGGGCTGG 2100
CTCACCCTGC CTTAAGGGCC GGCAGAAAGG GAGTTGAACA AACTGGCAGC 2150
TAGTGTGCAC TTGCTACCCA TAGCCGCTCT CGGCACCCAC AGACATCTGC 2200
CTGTGCTGCC CCGGGAGTAG GCAGTTGTCC AAGGGAGCCC TCTTGCATTA 2250
GGTGTCTGTG GCATGTCCCT AGGGTCCTGA CCTCATCCCT GAAGTTCCAC 2300
GGCAGCTGGT GAGTAACTGT GCCCAGCGGC TAGAGCAGGG ACCCTGCAAA 2350
GACCTCTTCC AGGAGCTGAC CCGGTAAGGC TCCATACCTC CTGGTCTGGA 2400
ATTGTTCCAG GGAAGGGGGC TTCTGTGGGC TCTGAAGTCT GTGACAAGCT 2450
CAGCTCCTCT TCCTGTCCGC GTTGTTGGAG TTTAGCAGTT GCCTCCCATC 2500
CCTGCTTCAG TGTAGAGTGC ATGCACCCTC CTGCTCAGGG CCTCAGTGAG 2550
AAGCTTGCCG AAGGAAAAGG ATGTTGCTTT GTGTCTGACC GGCATGTAGT 2600
GGGAGGCCTG TGCCTTCAGG CTGAGAATTG GCCTTGTTAG AGGCTCGCCT 2650
ACAGACTGAT CCTCTCTCAA CAGGCTGACC CACGAGTACC TGAGCACGGC 2700
CCCTTTTGCC GACTACCTCG ACAGCATCTA CTTCAACCGT TTTCTGCAGT 2750
GGAAGTGGCT GGAAAGGTGA ACGCCTCCCA AGCTGGCCTG TGGTAGGTCA 2800
GATTGTGGGC TCTCATGGCA AGCCCTGCCT GTGTAAATGT TTGTTTTTTT 2850

Fig 10G

```
ACTCTCGCCT ATAGGCAACC AGTGACCAAA AACACCTTCA GGCAGTACCG 2900
AGTCCTGGGC AAAGGTGGCT TTGGGGAGGT AAGTGGCCAC CCAGGTCTTT 2950
GCAGAGTATA GTAGATTGGG TACTTGTAGG CCCGGGCCCT CCCACCTTCA 3000
GCAAGAGCAT GGCTGGCCCC CATCCATCCC TGTCTGCCTG GTTACAGGTA 3050
TGTGCCTGCC AGGTGCGGGC AACAGGCAAG ATGTACGCAT GCAAGAAACT 3100
GGAAAAGAAG CGGATAAAGA AGCGAAAGGG GGAGGCCATG GCTCTCAACG 3150
AGAAACAGAT CTTGGAGAAA GTGAACAGTA GGTTTGTAGT AAGTACACAA 3200
GGAGCCCTCT CCCTTCCCCT GGGCCACACC ACCTGCTACA TTCCCCACCC 3250
ACCAGGCTAA ATTCCCTCCC TATTGCCAAA GGGACTGCCC TCTGCCCCCT 3300
CCCTTCCTGC CCTGGACAGT ACCCAAGAAG GGTGGGGTAG GAGTGCTATT 3350
CCAGGCTCAG GAAGCCTGCT GGGTCCTAAG GAGTGGCACA CAGAAGATGG 3400
TGGCTGCCAT TAGCATTTAG GAGAGTGAGC ATGCGTGCGA ACAGGTTCCT 3450
GGCTGAGGGT GTGGGTCTCC CGAGCACAGG TGAGCTTAGC CTACGCCTAT 3500
GAGACCAAGG ATGCACTGTG CCTGGTGCTG ACATTGATGA ATGGAGGTGA 3550
CCTAAAGTTC CACATCTACC ACATGGGCCA GGCTGGCTTT CCTGAAGCAC 3600
GTGCTGTCTT CTATGCTGCT GAGATCTGCT GTGGCCTGGA AGACCTGCAC 3650
CGGGAACGCA TTGTGTACAG GTAGGGCGGG GCTCGGGCGG CTGGCTGTTC 3700
CAGGTCTTGG TCCTTCCTGG ACAGCGACAG CAGTTAGGTT TGAGAGTGGC 3750
AGCTCTGGCT GCCTGTCAGG CTGGGCAGTG GAAGTATTGT GTGCTGGAGG 3800
TTTCTCTAAC CACAAGCCAA CACCGGCTCC ACACAAACCC CCTCGTGTTC 3850
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TGCCTTGCAG GCGGGCTCAG TAGTAGCGAG CCCTACTTAT GATTATGGAT 4050
GGAGCGTCTA TTAGAGCCTC CCAGTGGCCC TCTGAGGGGA TGGAAGGCTA 4100
GTCAGCTTAT AACTTCGTAT AGCATACATT ATACGAAGTT ATGCTAGCCT 4150
CATGTGACAG AGAAGGTAGA GTTGGCCCCT GGGGGAAGGG AGCACAGGTG 4200
AACAAGTCCA AGGATGACTT CCAGGGACCC CTCTCCCCAG GTGCTCCCCC 4250
ACTGGGTTGC AGTCCTCAGT TCTTACTACT CTAGAAAGTT CCTGAGGTCC 4300
TGGTTGCTGC CCGGGGAGAG CAGGGCTCGT TCACCTTGCT CCATGCCTGA 4350
ACCCAGTAGG CACTTGAACT GCCAGGGGCA GGCTCTGTCT GGCTTCCTGG 4400
AAAACAGGCC TCTGACCCTG TGTTTCTCTC CCCATAGGGA TCTAAAGCCA 4450
GAGAATATCC TTCTGGATGA CCATGGTGGG TGAGAAGGCC CAAGTGGGAG 4500
ATGGGGTGAG GTTGGAAGCT AGGCCTATCA GTGAACCCAC CTGCTTCCAT 4550
ACCCCTACCC AGGCCACATT CGGATCTCGG ACCTGGGACT GGCCGTGCAT 4600
GTGCCTGAGG GCCAGACCAT CAAAGGCCGT GTGGGCACTG TGGGCTACAT 4650
GGGTAAGTCC TGTTGACCTA GTACGCCAGT CTCCTCAGCC CCCTTGCTGT 4700
CCCAGGCCTG GCCTACTTAC TCACTGTTGT ACTAGGGGAG GGAGGGTGCT 4750
AACTTCAGAG AGTAGGTGTA GGTGTGTGTG TGTGTGTGTG TGTGTGTGTG 4800
TGTGTGTGTG TGTGATGTGG GGAAACCAAG ACTTAGCCAG ATCCCACCAT 4850
TGTGAACCCT GCTATTCATT TGCCGGATGC TTATTGAACA CCTAGGACTG 4900
GGTGGGCCTG TGAGGGTGAA GGGAAACAGA AACAGAGTAC ACATCCATGC 4950
CCCATGCTTG GATAGCATGC CATTCACAGG GGACACAGGC AGTGGAGACC 5000
TAGCTTGTGG ACAGTGTGAC ACAGTACAGT CTAGGCTCTC CAGAGTCCAT 5050
CTTGGGCTAG GCATTCCACT TCCTGCCTTC TTTCTCCTAC TGTAAAACCT 5100
CATCAGAGCT GCCTCCTGGG GGGCTTTGAG GTGAAAGGAG ATGCTAAATG 5150
GAGAACTGTG CATGGGGCCT GCCAGGGATG GTGGGAGGTA GCAGCCAGTG 5200
GGCGTTTAAA ATCGCTTGTG AAAGCCAAAA GTTACAAATC AGTAGCTACG 5250
TGGTCCTGAT CTGGCATACA TTAAATTTGA AGTTGAATTG TTTTTAGAAT 5300
AATTTGAATT GATTGCCAGC ATTTAAGGCA AACAACTTAA TGTATTAAAA 5350
AAAAAAAAAA AAAAAGGTGT ATGGGGTCTT GGTTGGGTGT GTATGATGTA 5400
ACTCAGAGTA CGTGCCTGAT AGTTGTGTGG CCCTGGGTTC AACAAACTCC 5450
AGCACTAGGA ATCCCACTCC TGCCCCCAGG GAAGACAAGC AATCAGACAC 5500
GGAGTTCCCT TGGTGTGACC CTCCCCTCAG GGCCACACCA GCCACCATTG 5550
ATGAGATGTG GCTTCTCATA CTGGCTCAGT CCACAGCAGG CCAGTGGCAT 5600
ACCTGCCTAT CCAGAGGATG TTTGATCAAA CTCTGGTTTT TGTTTCTGGG 5650
GCCCGAGGGC TCCCTGCTCC TCACGACCTG CCCGGTCCTG ACTCCTGGTC 5700
CCTGTCCAGC TCCAGAGGTG GTGAGGAATG AGCGCTACAC GTTCAGTCCT 5750
```

Fig 10H

GACTGGTGGG CGCTAGGCTG CCTCCTGTAC GAGATGATCG CAGGTCAGTC 5800
GCCCTTCCAG CAGAGGAAGA AGAAGATAAA GCGCGAAGAG GTGGAGCGGC 5850
TGGTCAAGGA AGTGGCCGAG GAGTACACAG ACCGCTTTTC CTCACAGGCG 5900
CGCTCACTCT GTTCTCAGGT ACAAGCCAGA GTCTTAGCTG GGGGCAGCTG 5950
GGGTACCTCA CCCACTCAGG TACCCTCACC ACCACTGCCC TTCTATAGCT 6000
TCTTAGCAAG GACCCTGCTG AGCGCCTGGG GTGTCGTGGA GGTGGCGCCC 6050
GTGAGGTAAA GGAGCACCCC CTTTTCAAGA AACTGAATTT CAAGCGGCTG 6100
GGAGCTGGCA TGCTAGAGCC ACCTTTTAAG CCTGATGTAA GTCCTGCCCT 6150
CCCTTGCTAG TGGCCAGCCG AGCCAGGGGG TGGGGCGGGG CAGGGCTGGG 6200
GTGGGTGACT GGGAGCAAGG GCCAAGTCAC TTTCAGTGCA GATGTTGGAA 6250
AAACCCCATC TAATGGCTTG GGCTGCCCCT CACCCCCAGT TTGTGTGTGT 6300
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTAGGCTTGT 6350
GAAAATGGAA TGCCCAACAT ACTGTGGGTT TTAGTGTACC AGGTTAGAAT 6400
GCTGGGCAGA CATGGAGATG CTCTGGGAGC ATTGCAAGAA CTCATGTCCT 6450
CTCTGTACTG TGATTGGTTA GCCTTCGATG GCAGGCATCT TTTCCCAGGG 6500
AGTGGCAATG GTGACCTAGT GGCTCAGGCT CATGTCTTCT TAGATAGCCA 6550
CCCAGTGGGC ACAGAGCAAG GTTTGTTCAC GAATTTGAAA TTAAATGCTC 6600
TGTTTGCTGA AGGGCCCCAC TGGACACCCA GATCACCCCT TCCTGGCCCA 6650
ATGGGAATAA CCCATTTCCA AAAGGAACTG AGACGGTGGT GCCAGCAGGG 6700
GCTGTTGGGC TAACTGCCCC TCTGCAGGCA GGCAATTACC CCCTTCAAAG 6750
TTTAACTTTA AAATATTTAT CTTATTCTTA ATTTTGTGTT TGTGTGTCTG 6800
TCTCTGTGTG GGTATGTATA TGTCTATGTA TGTGTGTATG TCTAGGAATG 6850
TGAATATGTC TATGTATGTG TGTGCCTGTG GAGCATGTGT ATGTCTGTAT 6900
GTGTGCGTCT GCGTGGGGAC GTGGATAGGT GTGCAGTTGC CACGGAGGCC 6950
CGAAGCTTTA GATCCTCCTG GGAATGGAGT CATGGGTGGT TGTGAGTGTC 7000
TGCATGACAT TAGTTTGAGG AATCAACTTA GATCCCCCAC AGCAGCAGTG 7050
CATACTTCTA ACTGCTGAGC CCGTCTTTCT AGTCCCAGAA TCTCCCTCTT 7100
GAGAGTTTTA CCTCTGAGCT AAGCATCTTG TGGATTGTAC ACCGTGCAGA 7150
CAATTGTACT CTAGATATTA GAGCCTAATA GCAGAAACCC TGCTCAGACT 7200
CACGTCAAGT CTGTTGGTTC ATTTGTCCCC AAGTCTCTCT GGGCCATCAC 7250
TGGGGCTTTG TGCTGGGTTG CTAAGACTGT CTCTTGGCTC TTCAACTTGT 7300
CTGTACAGAA CCTGAATTCA GGTTTCTCTG TAATGTCAGA GGAAGGGTTC 7350
TGGTTGGCCA GGATGGGGTC ATGTCCTTGC CTCCCTCCCT TAAGTTTGTG 7400
GACACGGGGG TCCTGTGATT TGACAGTTTG CCACTTGGGA AAGTCTTGAG 7450
GAGATGGGGG CACCAGGTTG ATGGCTGTCA GCCACATATG TTTATGCGTC 7500
TGACAAATAC CTGAAAATCT GTATGAGTCA GGTCTTTGAG ATAATGAGGT 7550
TCATAGAGCC AGGCCTATTC TCATACTATA GTGGAGAGAA ATAATGAAAA 7600
TAAACTAACA AGCAAAGAGT GTTTTCAGAT AGTCCCAAGT CTGACAGGAA 7650
AAAGAGCAGG CAAAGAAGGC AGTGATGTGG GTGTGGTCGT CTCTGAGAGA 7700
GTGATGTTTA CGCAGAACTG AAGGAGAGAG TCTCGCAAGA TGAGAGGCCA 7750
CAGGCATTAG AGGCAGAAGG GACAGGCAGG CACTGAGGGC CAGTGGCAGG 7800
GTCAGCCTAG GGACATGGAA CCACCACCAT GAGGCCATCG TGTCCGGAGC 7850
AAGTCAGTGC AGAGCAGCAA CAGTGAATGT CGCGATGTTC TAATGATGAC 7900
AGGAGACCTT CGGGCTGCAG CTCTAATGTG AGGTGTGGAA ATGGGTCATA 7950
GAGAATGAGT GAGCTGGGGA AAAGTCAGCG AGGGGGCCTC TGAGTGATGG 8000
GTCACACAAG TGAGGGTAGT GCCTCTGAGT GAGGACTCAC ACAGTGGGGG 8050
AGGCGTTCTG AGTGATGGCT CCCATGCTGC AGGTGAGCAC TGTCAATGAG 8100
TTGGCACTTG AACCGGACAG GTCTCACTTC ATCCTGCTGC TCCAGGCTGG 8150
AGCTACTGCA CAGGTTGACT CCTCTCTCTG TACATCATCT AAGTAATTGC 8200
ACGGCCCGTG GGTCATCTGT TCAAAGTCAG AAAATAAGGA CAGTGTCTTG 8250
GGACTTAGAA GTAGCAGATG AAAAAACAAA TTTAACAATG CTTTTATGGT 8300
TTTAATTTAA AAAAAAAAAT TACATGTCTA CCTATGTATC TGTGTGTGAG 8350
TGTGTGCATG TGAATTTAGG GGCCCCTGGA AGAACAGTGC AAGTTTTTAA 8400
CCTCTAGACC TGCACTTGAC AATATTGGGG GCGGGAGGGC GGGTTTCTCT 8450
GTGTAGCCCT GGCTGCTCTG GGACTCACTC TGTAGACCAG GCTGGCCTTG 8500
ACTCATAGAT CCACCTGCCT CTGCCTTCTG AGTGCTGGGA CCAAAGGTGT 8550
GCACTACCAT GCCCAGCCGA CATACATTTT TAAAAAAGAT TTATTTTATG 8600
TATATGAGTA CATTGTCACT GTCTTCAGAC ACACTGGAAG AGTGCATCAG 8650

Fig 10I

ATCTCATTAT	AGATGGTTGT	GAGCCACCAT	GTGGTTGCTG	GGAATTGAAC	8700
TCCGGATCTC	CAGAAAAGCA	GTCAGTGCTC	TTAACTACTG	AACCACCTCT	8750
CCAGCCCAAC	AACACATTTT	GAACAACTCT	GTTGAGATGA	AACTCACAGT	8800
CAATCCATAT	AAAGTATTCA	TGTCAACTGA	AAGAGTCACG	TGACTGTCAC	8850
CACAAAAGTT	CAGAATGTTT	TTGCCAGCCC	TTTCAGAACA	TAAGCAAAAC	8900
CTTATGTCCA	TTAGGGTTCC	AAATCCTTTC	CTCCTACCCC	AAGAAACCAC	8950
AGCTGTCTGT	CTTCAACTAG	TTTAGATGCT	TCAGTTGTGT	GGTCAGATGT	9000
GGGCATGCTG	AACACGGTCC	TCTGTGACTG	GCTTCTTTTG	CTAAGCATGG	9050
TTTTGTTTTG	TTTTGTTTTG	TTTTTAAGTT	TATCAAGACA	GGTTTTCTCT	9100
GTATAGTTCT	GGCCGTCCTG	GAACTCACTC	TGTAGACCAG	GCTGGCCTCC	9150
AACTCAGAAA	TCCACCTGCC	TCTGCCTCGC	TAAGCATGCT	TTTAAAGTTC	9200
ATCTATGTCG	GAGCATTCTT	CTTAAAAGCT	GGTTAATATT	CTGTAACGTG	9250
GATATAAAAT	AAACCTTATT	CGTTCCTGGT	TGACATGAGG	GTGTTTATCT	9300
TTTTGAGGGG	TGGGTCTATC	AGGAATAATC	CTGTCGGCTT	TTTTGAGATG	9350
AGGTGCCATT	GCATTGTCTT	GTCCAGTTTT	GAAATGGCAG	ACTCGGGCAT	9400
TCTTCCTGAC	CTCAGTATAT	ACATTGACGG	AACATTATGA	AGTCAAAAAT	9450
ACTGTATCTT	GTAGGGAGCT	TTAGACCTTT	CCAGGTTAGG	TACACCATTT	9500
TACATGATGG	TGATGATCAT	GTATGTATCA	GGCAGGTTGG	TAGGTATGTG	9550
TGTGTGTGTG	AGAGAGTGTG	TGTGTGTGTG	TGTGTGGTCT	ATGTGTGTGA	9600
GTGTGTGGTC	TATGTGTGTG	AGTGTGTGTG	TGTGAGTGTG	TGTGTGTGTG	9650
TGTGTGTATT	GAACCCAGGG	CCTAAAGCAT	ACTGAACCAC	TGTTCTATCA	9700
CTGAACTACA	CCTCTGACTT	CATGATGGTA	ATATGTTAAT	TGACGTCCTT	9750
TGTGGTAAGA	TTTTGAAAGC	AGCACAGATT	GAAAATAAGA	AAGCTTCAAA	9800
TACAGAAGCC	AGTTCCCAGC	AGGAACTGGG	AGGGCGGCTG	GTAAGACCAA	9850
TCCAGTACCG	GACATCCCAG	AGTTGAGACA	CGCACACACA	CACACACAGT	9900
CCCTATGGAG	GGAGCCAGGA	CTCCCCTTCC	CACCCCAACC	CCAACACATA	9950
CCCAGGCAGT	CCTGTGCAAG	TACAAGGTCA	GCCTAACACC	AGGTCTGCAC	10000
ACAGCCTGTC	CAGGCTCCGC	ACAGCGAGGG	TCACGATCCT	CGTCTATGGA	10050
GGCGAAAACT	GCCTCAGAGA	CAGTAGAGTG	CTGAGCTGAG	GAGTGTGTGG	10100
GGCACCATGC	TGATCTCTCC	AAATCAGGAT	GCTGCCAAGG	GTGGCAGACT	10150
CAAGCCCTCC	TGACTCCTGC	CTCTTCCACC	TTAGCCCCAG	GCTATTTATT	10200
GCAAGGATGT	CCTGGACATT	GAACAGTTCT	CTACAGTTAA	AGGTGTGGAT	10250
CTGGAGCCCA	CAGACCAAGA	CTTCTACCAG	AAGTTTGCCA	CAGGCAGTGT	10300
GTCCATCCCC	TGGCAGAATG	AGGTAGGGAT	TATCCTGTGA	CTGGGATGAG	10350
GTCCAGGTAG	GGCACCCCCA	GGGCCAACTC	TCACCGCTTC	CTGTTCCTGG	10400
GTAGATGGTG	GAGACCGAGT	GCTTCCAGGA	ACTCAATGTC	TTTGGGCTGG	10450
ATGGGTCTGT	TCCCCCAGAC	CTGGACTGGA	AGGGCCAGCC	CACTGCACCC	10500
CCCAAGAAGG	GATTGCTACA	GAGACTCTTC	AGTCGCCAAG	TAAGTCCTAG	10550
CAGTGTCTGC	CCTGGGTCCC	ACCTGCTCCA	CTGGGAGAGT	GGGTGGGAGG	10600
CAGAGCTGGT	GCATGCCTGC	CCCGGGAGTG	GGCATCTTCC	TGTGGTGCCC	10650
AAGGTGCCTC	ATTCCTGCCT	GTCCCCCCTG	ACCCCCACGC	CTGGCTGGTC	10700
TCATGGCCTC	TTTTCTCTTT	CCAGAGGTGA	GCAGTGTGGG	CTCCCCATGG	10750
GTTGGCCTTG	CTGCCTGACT	GTGGGGAGCC	CCAGGCAGCC	CATCCGAGGC	10800
AGAGGCGAGA	TGTAGGAGAA	AGAAGTCTGG	TGTCCACCTT	ACCCGCTCCT	10850
TCCCTGCCGC	TTGCCTCCCT	GCACCCTGAC	CCCTTCAAGC	TGCTAACCTT	10900
GCTTAGCAAC	TGTCTCCTTG	TGCCAGAGTC	TGTCCAGGGG	GATGGACAGA	10950
GGCCTTGCTC	TGACAGATCG	AGTCGACGCC	CTATAGTGAG	TCGTATTAGA	11000
GCTCGCGGCC	GC				11012

Fig 10J

GRK6/del allele Sequence

```
         10        20         30        40        50         60        70        80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
TCTAGAACTGACTTGTAGACCAGGC TGGCCCCAAACTCAGAGATCCACCT GTGTTTTGAATGGCAAGTGCTACCA TGCCCAGCTTCTTGTCTTGTCTACT   100

TCTTTTTGTTTTTCTTCTTTTTTTT TTTTTTTCCAAAACATAATTCCTCT GTGTAGCCCTGGCTGTCCTGGAAGT TGCTCTGCCAACCAGGCTGACCTTG   200

AACTCAGATTTACCTGTGTCTGCCT CCCACGTGCTGGGATTAAAGGTGTG CCCCACCACTGCCCAGCTCCTATTC TCCTAACCTGTAGACTTCCCACTGT   300

GTTAGGAGTGAATGAGGCGGAACTT CTTGATGAGATGTCCTCATTGGTCA TTTTGGTTCTCATCCAGGGAACTCT TACCATGGGTGCCCACAAGGGCCAT   400

GTGTGTCCTGGAACTTGTTAAGGGC ATGCTGGAATGTTTGGAAGAAGCCT CAAGGTTCCTCCCGCAGCAGGTTTG GCCTTACTTAACAGGGCCCTGAAGG   500

CCTCTCTGTACAACATGTTTAGGGG AAGGTTCCGAGGCAGGCGGCTCAGG ACTCAATGGGACCCAGTTCCTGATT GCTCTTGCAGGTGGTGGCGGGAATC   600
                                                                                             G  G  G  N  R
GCAAAGGCAAGAGCAAGAAATGGCG CCAGATGCTGCAGTTCCCCCATATC AGCCAGTGTGAGGAGCTTCGACTCA GCCTTGGTGAGGCCTGGCTCCCAGA   700
 K  G  K  S  K  K  W  R    Q  M  L  Q  F  P  H  I    S  Q  C  E  E  L  R  L  S   L  G
GCAGACTGGGGGGAGGGGAGCTGGG GGGGGTAGCCATGAGGAGTCATCCC CAGACATATCCTTGGCCATGGGGGC CTGGGGGGGGGGGAATTCAGGAAGA   800

CCGAGACGCCATAGTCCAGTTTCAG TTCCTGGGCCTGAAATGGCAGAGGG CAGAGAACGGAGACTGGTGTCAGCA GAGTGGGCATGGGCGAAGGCAGAGG   900

GCCAGGATGGAGGGAGGTGAGAGAA ACTTAGGAGGGAAGTCAGGTTTGGA ACACCAGTCCTGCGCTCATGAGCTT TGCACCTGAGGGTACCCATCAAAGC  1000

TAGGGCAGTATGGTGGTTACTTCTA GAAGGGCTCATCCCAGTTCCAGCAT CGTGGATGAGGGTCTGAACTGTATG TACCTCAGGTGGCCCCGTCCTCTCC  1100

TGATGGCTTGCATGCGCTTGGGACG GAGGCCCAGCTGAGAGCAGCGGAAG CCACAGCTTCCTCTCGCACTTTCGC CAGGCAGGCAACAGGTGTAGACTTG  1200

GGCTGGGCTGGGCGGCAACCACACA GACCACACCCAACTCCAGAACTGTG ACAAGCAAGTCCAGGAAACAACAGG GAAAGGAGACCTGGTGTCTAGAGGC  1300

GCGCCATAACTTCGTATAGCATACA TTATACGAAGTTATGCTAGCCTCAT GTGACAGAGAAGGTAGAGTTGGCCC CTGGGGGAAGGGAGCACAGGTGAAC  1400

AAGTCCAAGGATGACTTCCAGGGAC CCCTCTCCCCAGGTGCTCCCCCACT GGGTTGCAGTCCTCAGTTCTTACTA CTCTAGAAAGTTCCTGAGGTCCTGG  1500

TTGCTGCCCGGGGAGAGCAGGGCTC GTTCACCTTGCTCCATGCCTGAACC CAGTAGGCACTTGAACTGCCAGGGG CAGGCTCTGTCTGGCTTCCTGGAAA  1600

ACAGGCCTCTGACCCTGTGTTTCTC TCCCCATAGGGATCTAAAGCCAGAG AATATCCTTCTGGATGACCATGGTG GGTGAGAAGGCCCAAGTGGGAGATG  1700
                                    D  L  K  P  E   N  I  L  L  D  D  H  G
GGGTGAGGTTGGAAGCTAGGCCTAT CAGTGAACCCACCTGCTTCCATACC CCTACCCAGGCCACATTCGGATCTC GGACCTGGGACTGGCCGTGCATGTG  1800
                                                              H  I  R  I  S   D  L  G  L  A  V  H  V
CCTGAGGGCCAGACCATCAAAGGCC GTGTGGGCACTGTGGGCTACATGGG TAAGTCCTGTTGACCTAGTACGCCA GTCTCCTCAGCCCCCTTGCTGTCCC  1900
P  E  G  Q  T  I  K  G  R   V  G  T  V  G  Y  M  G
AGGCCTGGCCTACTTACTCACTGTT GTACTAGGGGAGGGAGGGTGCTAAC TTCAGAGAGTAGGTGTAGGTGTGTG TGTGTGTGTGTGTGTGTGTGTGTGT  2000

GTGTGTGTGTGATGTGGGGAAACCA AGACTTAGCCAGATCCCACCATTGT GAACCCTGCTATTCATTTGCCGGAT GCTTATTGAACACCTAGGACTGGGT  2100

GGGCCTGTGAGGGTGAAGGGAAACA GAAACAGAGTACACATCCATGCCCC ATGCTTGGATAGCATGCCATTCACA GGGGACACAGGCAGTGGAGACCTAG  2200

CTTGTGGACAGTGTGACACAGTACA GTCTAGGCTCTCCAGAGTCCATCTT GGGCTAGGCATTCCACTTCCTGCCT TCTTTCTCCTACTGTAAAACCTCAT  2300

CAGAGCTGCCTCCTGGGGGGCTTTG AGGTGAAAGGAGATGCTAAATGGAG AACTGTGCATGGGGCCTGCCAGGGA TGGTGGAGGTAGCAGCCAGTGGGC   2400

GTTTAAAATCGCTTGTGAAAGCCAA AAGTTACAAATCAGTAGCTACGTGG TCCTGATCTGGCATACATTAAATTT GAAGTTGAATTGTTTTTAGAATAAT  2500

TTGAATTGATTGCCAGCATTTAAGG CAAACAACTTAATGTATTAAAAAAA AAAAAAAAAAAAGGTGTATGGGGTC TTGGTTGGGTGTGTATGATGTAACT  2600

CAGAGTACGTGCCTGATAGTTGTGT GGCCCTGGGTTCAACAAACTCCAGC ACTAGGAATCCCACTCCTGCCCCCA GGGAAGACAAGCAATCAGACACGGA  2700

GTTCCCTTGGTGTGACCCTCCCCTC AGGGCCACACCAGCCACCATTGATG AGATGTGGCTTCTCATACTGGCTCA GTCCACAGCAGGCCAGTGGCATACC  2800
```

Fig 10K

GRK6/del allele Sequence

```
         10        20        30        40        50        60        70        80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
TGCCTATCCAGAGGATGTTTGATCA AACTCTGGTTTTTGTTTCTGGGGCC CGAGGGCTCCCTGCTCCTCACGACC TGCCCGGTCCTGACTCCTGGTCCCT   2900

GTCCAGCTCCAGAGGTGGTGAGGAA TGAGCGCTACACGTTCAGTCCTGAC TGGTGGGCGCTAGGCTGCCTCCTGT ACGAGATGATCGCAGGTCAGTCGCC   3000
       P  E  V  V  R  N   E  R  Y  T  F  S  P  D   W  W  A  L  G  C  L  L  Y   E  M  I  A  G  Q  S  P
CTTCCAGCAGAGGAAGAAGAAGATA AAGCGCGAAGAGGTGGAGCGGCTGG TCAAGGAAGTGGCCGAGGAGTACAC AGACCGCTTTTCCTCACAGGCGCGC   3100
 F  Q  Q  R  K  K  K  I   K  R  E  E  V  E  R  L  V   K  E  V  A  E  E  Y  T   D  R  F  S  S  Q  A  R
TCACTCTGTTCTCAGGTACAAGCCA GAGTCTTAGCTGGGGGCAGCTGGGG TACCTCACCCACTCAGGTACCCTCA CCACCACTGCCCTTCTATAGCTTCT   3200
S  L  C  S  Q                                                                                     L  L
TAGCAAGGACCCTGCTGAGCGCCTG GGGTGTCGTGGAGGTGGCGCCCGTG AGGTAAAGGAGCACCCCCTTTTCAA GAAACTGAATTTCAAGCGGCTGGGA   3300
 S  K  D  P  A  E  R  L   G  C  R  G  G  G  A  R  E   V  K  E  H  P  L  F  K   K  L  N  F  K  R  L  G
GCTGGCATGCTAGAGCCACCTTTTA AGCCTGATGTAAGTCCTGCCCTCCC TTGCTAGTGGCCAGCCGAGCCAGGG GGTGGGGCGGGGCAGGGCTGGGGTG   3400
A  G  M  L  E  P  P  F  K   P  D
GGTGACTGGGAGCAAGGGCCAAGTC ACTTTCAGTGCAGATGTTGGAAAAA CCCCATCTAATGGCTTGGGCTGCCC CTCACCCCCAGTTTGTGTGTGTGTG   3500

TGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTAGGCTTGTGAA AATGGAATGCCCAACATACTGTGGG TTTTAGTGTACCAGGTTAGAATGCT   3600

GGGCAGACATGGAGATGCTCTGGGA GCATTGCAAGAACTCATGTCCTCTC TGTACTGTGATTGGTTAGCCTTCGA TGGCAGGCATCTTTTCCCAGGGAGT   3700

GGCAATGGTGACCTAGTGGCTCAGG CTCATGTCTTCTTAGATAGCCACCC AGTGGGCACAGAGCAAGGTTTGTTC ACGAATTTGAAATTAAATGCTCTGT   3800

TTGCTGAAGGGCCCCACTGGACACC CAGATCACCCCTTCCTGGCCCAATG GGAATAACCCATTTCCAAAAGGAAC TGAGACGGTGGTGCCAGCAGGGGCT   3900

GTTGGGCTAACTGCCCCTCTGCAGG CAGGCAATTACCCCCTTCAAAGTTT AACTTTAAAATATTTATCTTATTCT TAATTTTGTGTTTGTGTGTCTGTCT   4000

CTGTGTGGGTATGTATATGTCTATG TATGTGTGTATGTCTAGGAATGTGA ATATGTCTATGTATGTGTGTGCCTG TGGAGCATGTGTATGTCTGTATGTG   4100

TGCGTCTGCGTGGGGACGTGGATAG GTGTGCAGTTGCCACGGAGGCCCGA AGCTTTAGATCCTCCTGGGAATGGA GTCATGGGTGGTTGTGAGTGTCTGC   4200

ATGACATTAGTTTGAGGAATCAACT TAGATCCCCCACAGCAGCAGTGCAT ACTTCTAACTGCTGAGCCCGTCTTT CTAGTCCCAGAATCTCCCTCTTGAG   4300

AGTTTTACCTCTGAGCTAAGCATCT TGTGGATTGTACACCGTGCAGACAA TTGTACTCTAGATATTAGAGCCTAA TAGCAGAAACCCTGCTCAGACTCAC   4400

GTCAAGTCTGTTGGTTCATTTGTCC CCAAGTCTCTCTGGGCCATCACTGG GGCTTTGTGCTGGGTTGCTAAGACT GTCTCTTGGCTCTTCAACTTGTCTG   4500

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ACGGGGGTCCTGTGATTTGACAGTT TGCCACTTGGGAAAGTCTTGAGGAG ATGGGGGCACCAGGTTGATGGCTGT CAGCCACATATGTTTATGCGTCTGA   4700

CAAATACCTGAAAATCTGTATGAGT CAGGTCTTTGAGATAATGAGGTTCA TAGAGCCAGGCCTATTCTCATACTA TAGTGGAGAGAAATAATGAAAATAA   4800

ACTAACAAGCAAAGAGTGTTTTCAG ATAGTCCCAAGTCTGACAGGAAAAA GAGCAGGCAAAGAAGGCAGTGATGT GGGTGTGGTCGTCTCTGAGAGAGTG   4900

ATGTTTACGCAGAACTGAAGGAGAG AGTCTCGCAAGATGAGAGGCCACAG GCATTAGAGGCAGAAGGGACAGGCA GGCACTGAGGGCCAGTGGCAGGGTC   5000

AGCCTAGGGACATGGAACCACCACC ATGAGGCCATCGTGTCCGGAGCAAG TCAGTGCAGAGCAGCAACAGTGAAT GTCGCGATGTTCTAATGATGACAGG   5100

AGACCTTCGGGCTGCAGCTCTAATG TGAGGTGTGGAAATGGGTCATAGAG AATGAGTGAGCTGGGGAAAAGTCAG CGAGGGGGCCTCTGAGTGATGGGTC   5200

ACACAAGTGAGGGTAGTGCCTCTGA GTGAGGACTCACACAGTGGGGGAGG CGTTCTGAGTGATGGCTCCCATGCT GCAGGTGAGCACTGTCAATGAGTTG   5300

GCACTTGAACCGGACAGGTCTCACT TCATCCTGCTGCTCCAGGCTGGAGC TACTGCACAGGTTGACTCCTCTCTC TGTACATCATCTAAGTAATTGCACG   5400

GCCCGTGGGTCATCTGTTCAAAGTC AGAAAATAAGGACAGTGTCTTGGGA CTTAGAAGTAGCAGATGAAAAAACA AATTTAACAATGCTTTTATGGTTTT   5500

AATTTAAAAAAAAAAATTACATGTC TACCTATGTATCTGTGTGTGAGTGT GTGCATGTGAATTTAGGGGCCCCTG GAAGAACAGTGCAAGTTTTTAACCT   5600
```

Fig 10L

GRK6/del allele Sequence

```
        10        20        30        40        50        60        70        80        90       100
1234567890123456789012345 6789012345678901234567890 1234567890123456789012345 6789012345678901234567890
CTAGACCTGCACTTGACAATATTGG GGGCGGGAGGGCGGGTTTCTCTGTG TAGCCCTGGCTGCTCTGGGACTCAC TCTGTAGACCAGGCTGGCCTTGACT   5700

CATAGATCCACCTGCCTCTGCCTTC TGAGTGCTGGGACCAAAGGTGTGCA CTACCATGCCCAGCCGACATACATT TTTAAAAAAGATTTATTTTATGTAT   5800

ATGAGTACATTGTCACTGTCTTCAG ACACACTGGAAGAGTGCATCAGATC TCATTATAGATGGTTGTGAGCCACC ATGTGGTTGCTGGGAATTGAACTCC   5900

GGATCTCCAGAAAAGCAGTCAGTGC TCTTAACTACTGAACCACCTCTCCA GCCCAACAACACATTTTGAACAACT CTGTTGAGATGAAACTCACAGTCAA   6000

TCCATATAAAGTATTCATGTCAACT GAAAGAGTCACGTGACTGTCACCAC AAAAGTTCAGAATGTTTTTGCCAGC CCTTTCAGAACATAAGCAAAACCTT   6100

ATGTCCATTAGGGTTCCAAATCCTT TCCTCCTACCCCAAGAAACCACAGC TGTCTGTCTTCAACTAGTTTAGATG CTTCAGTTGTGTGGTCAGATGTGGG   6200

CATGCTGAACACGGTCCTCTGTGAC TGGCTTCTTTTGCTAAGCATGGTTT TGTTTTGTTTTGTTTTGTTTTTAAG TTTATCAAGACAGGTTTTCTCTGTA   6300

TAGTTCTGGCCGTCCTGGAACTCAC TCTGTAGACCAGGCTGGCCTCCAAC TCAGAAATCCACCTGCCTCTGCCTC GCTAAGCATGCTTTTAAAGTTCATC   6400

TATGTCGGAGCATTCTTCTTAAAAG CTGGTTAATATTCTGTAACGTGGAT ATAAAATAAACCTTATTCGTTCCTG GTTGACATGAGGGTGTTTATCTTTT   6500

TGAGGGGTGGGTCTATCAGGAATAA TCCTGTCGGCTTTTTTGAGATGAGG TGCCATTGCATTGTCTTGTCCAGTT TTGAAATGGCAGACTCGGGCATTCT   6600

TCCTGACCTCAGTATATACATTGAC GGAACATTATGAAGTCAAAAATACT GTATCTTGTAGGGAGCTTTAGACCT TTCCAGGTTAGGTACACCATTTTAC   6700

ATGATGGTGATGATCATGTATGTAT CAGGCAGGTTGGTAGGTATGTGTGT GTGTGTGAGAGAGTGTGTGTGTGTG TGTGTGTGGTCTATGTGTGTGAGTG   6800

TGTGGTCTATGTGTGTGAGTGTGTG TGTGTGAGTGTGTGTGTGTGTGTGT GTGTATTGAACCCAGGGCCTAAAGC ATACTGAACCACTGTTCTATCACTG   6900

AACTACACCTCTGACTTCATGATGG TAATATGTTAATTGACGTCCTTTGT GGTAAGATTTTGAAAGCAGCACAGA TTGAAAATAAGAAAGCTTCAAATAC   7000

AGAAGCCAGTTCCCAGCAGGAACTG GGAGGGCCGCTGGTAAGACCAATCC AGTACCGGACATCCCAGAGTTGAGA CACGCACACACACACACACAGTCCC   7100

TATGGAGGGAGCCAGGACTCCCCTT CCCACCCCAACCCCAACACATACCC AGGCAGTCCTGTGCAAGTACAAGGT CAGCCTAACACCAGGTCTGCACACA   7200

GCCTGTCCAGGCTCCGCACAGCGAG GGTCACGATCCTCGTCTATGGAGGC GAAAACTGCCTCAGAGACAGTAGAG TGCTGAGCTGAGGAGTGTGTGGGGC   7300

ACCATGCTGATCTCTCCAAATCAGG ATGCTGCCAAGGGTGGCAGACTCAA GCCCTCCTGACTCCTGCCTCTTCCA CCTTAGCCCCAGGCTATTTATTGCA   7400
                                                                                      P  Q  A  I  Y  C  K
AGGATGTCCTGGACATTGAACAGTT CTCTACAGTTAAAGGTGTGGATCTG GAGCCCACAGACCAAGACTTCTACC AGAAGTTTGCCACAGGCAGTGTGTC   7500
 D  V  L  D  I  E  Q  F   S  T  V  K  G  V  D  L   E  P  T  D  Q  D  F  Y  Q   K  F  A  T  G  S  V  S
CATCCCCTGGCAGAATGAGGTAGGG ATTATCCTGTGACTGGATGAGGTC CAGGTAGGGCACCCCCAGGGCCAAC TCTCACCGCTTCCTGTTCCTGGGTA   7600
 I  P  W  Q  N  E
GATGGTGGAGACCGAGTGCTTCCAG GAACTCAATGTCTTTGGGCTGGATG GGTCTGTTCCCCCAGACCTGGACTG GAAGGGCCAGCCCACTGCACCCCCC   7700
 M  V  E  T  E  C  F  Q   E  L  N  V  F  G  L  D  G   S  V  P  P  D  L  D  W   K  G  Q  P  T  A  P  P
AAGAAGGGATTGCTACAGAGACTCT TCAGTCGCCAAGTAAGTCCTAGCAG TGTCTGCCCTGGGTCCCACCTGCTC CACTGGGAGAGTGGGTGGAGGCAG   7800
K  K  G  L  L  Q  R  L  F   S  R  Q
AGCTGGTGCATGCCTGCCCCGGGAG TGGGCATCTTCCTGTGGTGCCCAAG GTGCCTCATTCCTGCCTGTCCCCCC TGACCCCCACGCCTGGCTGGTCTCA   7900

TGGCCTCTTTTCTCTTTCCAGAGGT GAGCAGTGTGGGCTCCCCATGGGTT GGCCTTGCTGCCTGACTGTGGGGAG CCCCAGGCAGCCCATCCGAGGCAGA   8000
                  R  .
GGCGAGATGTAGGAGAAAGAAGTCT GGTGTCCACCTTACCCGCTCCTTCC CTGCCGCTTGCCTCCCTGCACCCTG ACCCCTTCAAGCTGCTAACCTTGCT   8100

TAGCAACTGTCTCCTTGTGCCAGAG TCTGTCCAGGGGGATGGACAGAGGC CTTGCTCTGACAGATCGAGTCGACG CCCTATAGTGAGTCGTATTAGAGCT   8200

CGCGGCCGC                                                                                                   8209
```

Fig 10M

GRK6/flox construct Feature Table

Key	From	To	Strand	Shown	Description
misc_feat...	1	6	5'	Boxed/N..	XbaI site
exon	586	681	5'	AA1/Fat...	exon 2 /codon_start...
misc_feat...	1292	1297	5'	Boxed/N...	XbaI site
misc_feat...	1339	1306	3'	Underli...	loxP site
misc_feat...	1340	1345	5'	Boxed/N...	NheI site
exon	1635	1672	5'	AA1/Fat...	exon 10 /codon_star...
exon	1760	1849	5'	AA1/Fat...	exon 11 /codon_star...
exon	2907	3115	5'	AA1/Fat...	exon 12 /codon_star...
exon	3196	3333	5'	AA1/Fat...	exon 13 /codon_star...
exon	7382	7519	5'	AA1/Fat...	exon 14 /codon_star...
exon	7602	7736	5'	AA1/Fat...	exon 15 /codon_star...
exon	7922	7927	5'	AA1/Fat...	exon 16-C /codon_st...
misc_feat...	8209	8202	3'	Boxed/B...	NotI site (from gen...

Fig 10N

SEQ ID No:2

```
TCTAGAACTG ACTTGTAGAC CAGGCTGGCC CCAAACTCAG AGATCCACCT     50
GTGTTTTGAA TGGCAAGTGC TACCATGCCC AGCTTCTTGT CTTGTCTACT    100
TCTTTTTGTT TTTCTTCTTT TTTTTTTTTT TTCCAAAACA TAATTCCTCT    150
GTGTAGCCCT GGCTGTCCTG GAAGTTGCTC TGCCAACCAG GCTGACCTTG    200
AACTCAGATT TACCTGTGTC TGCCTCCCAC GTGCTGGGAT TAAAGGTGTG    250
CCCCACCACT GCCCAGCTCC TATTCTCCTA ACCTGTAGAC TTCCCACTGT    300
GTTAGGAGTG AATGAGGCGG AACTTCTTGA TGAGATGTCC TCATTGGTCA    350
TTTTGGTTCT CATCCAGGGA ACTCTTACCA TGGGTGCCCA CAAGGGCCAT    400
GTGTGTCCTG GAACTTGTTA AGGGCATGCT GGAATGTTTG GAAGAAGCCT    450
CAAGGTTCCT CCCGCAGCAG GTTTGGCCTT ACTTAACAGG GCCCTGAAGG    500
CCTCTCTGTA CAACATGTTT AGGGGAAGGT TCCGAGGCAG GCGGCTCAGG    550
ACTCAATGGG ACCCAGTTCC TGATTGCTCT TGCAGGTGGT GGCGGGAATC    600
GCAAAGGCAA GAGCAAGAAA TGGCGCCAGA TGCTGCAGTT CCCCCATATC    650
AGCCAGTGTG AGGAGCTTCG ACTCAGCCTT GGTGAGGCCT GGCTCCCAGA    700
GCAGACTGGG GGGAGGGGAG CTGGGGGGGG TAGCCATGAG GAGTCATCCC    750
CAGACATATC CTTGGCCATG GGGGCCTGGG GGGGGGGGAA TTCAGGAAGA    800
CCGAGACGCC ATAGTCCAGT TTCAGTTCCT GGGCCTGAAA TGGCAGAGGG    850
CAGAGAACGG AGACTGGTGT CAGCAGAGTG GGCATGGGCG AAGGCAGAGG    900
GCCAGGATGG AGGGAGGTGA GAGAAACTTA GGAGGGAAGT CAGGTTTGGA    950
ACACCAGTCC TGCGCTCATG AGCTTTGCAC CTGAGGGTAC CCATCAAAGC   1000
TAGGGCAGTA TGGTGGTTAC TTCTAGAAGG GCTCATCCCA GTTCCAGCAT   1050
CGTGGATGAG GGTCTGAACT GTATGTACCT CAGGTGGCCC CGTCCTCTGC   1100
TGATGGCTTG CATGCGCTTG GGACGGAGGC CCAGCTGAGA GCAGCGGAAG   1150
CCACAGCTTC CTCTCGCACT TTCGCCAGGC AGGCAACAGG TGTAGACTTG   1200
GGCTGGGCTG GGCGGCAACC ACACAGACCA CACCCAACTC CAGAACTGTG   1250
ACAAGCAAGT CCAGGAAACA ACAGGGAAAG GAGACCTGGT GTCTAGAGGC   1300
GCGCCATAAC TTCGTATAGC ATACATTATA CGAAGTTATG CTAGCCTCAT   1350
GTGACAGAGA AGGTAGAGTT GGCCCCTGGG GGAAGGGAGC ACAGGTGAAC   1400
AAGTCCAAGG ATGACTTCCA GGGACCCCTC TCCCCAGGTG CTCCCCCACT   1450
GGGTTGCAGT CCTCAGTTCT TACTACTCTA GAAAGTTCCT GAGGTCCTGG   1500
TTGCTGCCCG GGGAGAGCAG GGCTCGTTCA CCTTGCTCCA TGCCTGAACC   1550
CAGTAGGCAC TTGAACTGCC AGGGGCAGGC TCTGTCTGGC TTCCTGGAAA   1600
ACAGGCCTCT GACCCTGTGT TTCTCTCCCC ATAGGGATCT AAAGCCAGAG   1650
AATATCCTTC TGGATGACCA TGGTGGGTGA GAAGGCCCAA GTGGGAGATG   1700
GGGTGAGGTT GGAAGCTAGG CCTATCAGTG AACCCACCTG CTTCCATACC   1750
CCTACCCAGG CCACATTCGG ATCTCGGACC TGGGACTGGC CGTGCATGTG   1800
CCTGAGGGCC AGACCATCAA AGGCCGTGTG GGCACTGTGG GCTACATGGG   1850
TAAGTCCTGT TGACCTAGTA CGCCAGTCTC CTCAGCCCCC TTGCTGTCCC   1900
AGGCCTGGCC TACTTACTCA CTGTTGTACT AGGGGAGGGA GGGTGCTAAC   1950
TTCAGAGAGT AGGTGTAGGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT   2000
GTGTGTGTGT GATGTGGGGA AACCAAGACT TAGCCAGATC CCACCATTGT   2050
GAACCCTGCT ATTCATTTGC CGGATGCTTA TTGAACACCT AGGACTGGGT   2100
GGGCCTGTGA GGGTGAAGGG AAACAGAAAC AGAGTACACA TCCATGCCCC   2150
ATGCTTGGAT AGCATGCCAT TCACAGGGGA CACAGGCAGT GGAGACCTAG   2200
CTTGTGGACA GTGTGACACA GTACAGTCTA GGCTCTCCAG AGTCCATCTT   2250
GGGCTAGGCA TTCCACTTCC TGCCTTCTTT CTCCTACTGT AAAACCTCAT   2300
CAGAGCTGCC TCCTGGGGGG CTTTGAGGTG AAAGGAGATG CTAAATGGAG   2350
AACTGTGCAT GGGGCCTGCC AGGGATGGTG GGAGGTAGCA GCCAGTGGGC   2400
GTTTAAAATC GCTTGTGAAA GCCAAAAGTT ACAAATCAGT AGCTACGTGG   2450
TCCTGATCTG GCATACATTA AATTTGAAGT TGAATTGTTT TTAGAATAAT   2500
TTGAATTGAT TGCCAGCATT TAAGGCAAAC AACTTAATGT ATTAAAAAAA   2550
AAAAAAAAAA AAGGTGTATG GGGTCTTGGT TGGGTGTGTA TGATGTAACT   2600
CAGAGTACGT GCCTGATAGT TGTGTGGCCC TGGGTTCAAC AAACTCCAGC   2650
ACTAGGAATC CCACTCCTGC CCCCAGGGAA GACAAGCAAT CAGACACGGA   2700
GTTCCCTTGG TGTGACCCTC CCCTCAGGGC CACACCAGCC ACCATTGATG   2750
AGATGTGGCT TCTCATACTG GCTCAGTCCA CAGCAGGCCA GTGGCATACC   2800
```

Fig 10 O

TGCCTATCCA GAGGATGTTT GATCAAACTC TGGTTTTTGT TTCTGGGGCC 2850
CGAGGGCTCC CTGCTCCTCA CGACCTGCCC GGTCCTGACT CCTGGTCCCT 2900
GTCCAGCTCC AGAGGTGGTG AGGAATGAGC GCTACACGTT CAGTCCTGAC 2950
TGGTGGGCGC TAGGCTGCCT CCTGTACGAG ATGATCGCAG GTCAGTCGCC 3000
CTTCCAGCAG AGGAAGAAGA AGATAAAGCG CGAAGAGGTG GAGCGGCTGG 3050
TCAAGGAAGT GGCCGAGGAG TACACAGACC GCTTTTCCTC ACAGGCGCGC 3100
TCACTCTGTT CTCAGGTACA AGCCAGAGTC TTAGCTGGGG GCAGCTGGGG 3150
TACCTCACCC ACTCAGGTAC CCTCACCACC ACTGCCCTTC TATAGCTTCT 3200
TAGCAAGGAC CCTGCTGAGC GCCTGGGGTG TCGTGGAGGT GGCGCCCGTG 3250
AGGTAAAGGA GCACCCCCTT TTCAAGAAAC TGAATTTCAA GCGGCTGGGA 3300
GCTGGCATGC TAGAGCCACC TTTTAAGCCT GATGTAAGTC CTGCCCTCCC 3350
TTGCTAGTGG CCAGCCGAGC CAGGGGGTGG GGCGGGGCAG GGCTGGGGTG 3400
GGTGACTGGG AGCAAGGGCC AAGTCACTTT CAGTGCAGAT GTTGGAAAAA 3450
CCCCATCTAA TGGCTTGGGC TGCCCCTCAC CCCCAGTTTG TGTGTGTGTG 3500
TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTA GGCTTGTGAA 3550
AATGGAATGC CCAACATACT GTGGGTTTTA GTGTACCAGG TTAGAATGCT 3600
GGGCAGACAT GGAGATGCTC TGGGAGCATT GCAAGAACTC ATGTCCTCTC 3650
TGTACTGTGA TTGGTTAGCC TTCGATGGCA GGCATCTTTT CCCAGGGAGT 3700
GGCAATGGTG ACCTAGTGGC TCAGGCTCAT GTCTTCTTAG ATAGCCACCC 3750
AGTGGGCACA GAGCAAGGTT TGTTCACGAA TTTGAAATTA AATGCTCTGT 3800
TTGCTGAAGG GCCCCACTGG ACACCCAGAT CACCCCTTCC TGGCCCAATG 3850
GGAATAACCC ATTTCCAAAA GGAACTGAGA CGGTGGTGCC AGCAGGGGCT 3900
GTTGGGCTAA CTGCCCCTCT GCAGGCAGGC AATTACCCCC TTCAAAGTTT 3950
AACTTTAAAA TATTTATCTT ATTCTTAATT TTGTGTTTGT GTGTCTGTCT 4000
CTGTGTGGGT ATGTATATGT CTATGTATGT GTGTATGTCT AGGAATGTGA 4050
ATATGTCTAT GTATGTGTGT GCCTGTGGAG CATGTGTATG TCTGTATGTG 4100
TGCGTCTGCG TGGGGACGTG GATAGGTGTG CAGTTGCCAC GGAGGCCCGA 4150
AGCTTTAGAT CCTCCTGGGA ATGGAGTCAT GGGTGGTTGT GAGTGTCTGC 4200
ATGACATTAG TTTGAGGAAT CAACTTAGAT CCCCCACAGC AGCAGTGCAT 4250
ACTTCTAACT GCTGAGCCCG TCTTTCTAGT CCCAGAATCT CCCTCTTGAG 4300
AGTTTTACCT CTGAGCTAAG CATCTTGTGG ATTGTACACC GTGCAGACAA 4350
TTGTACTCTA GATATTAGAG CCTAATAGCA GAAACCCTGC TCAGACTCAC 4400
GTCAAGTCTG TTGGTTCATT TGTCCCCAAG TCTCTCTGGG CCATCACTGG 4450
GGCTTTGTGC TGGGTTGCTA AGACTGTCTC TTGGCTCTTC AACTTGTCTG 4500
TACAGAACCT GAATTCAGGT TTCTCTGTAA TGTCAGAGGA AGGGTTCTGG 4550
TTGGCCAGGA TGGGGTCATG TCCTTGCCTC CCTCCCTTAA GTTTGTGGAC 4600
ACGGGGGTCC TGTGATTTGA CAGTTTGCCA CTTGGGAAAG TCTTGAGGAG 4650
ATGGGGGCAC CAGGTTGATG GCTGTCAGCC ACATATGTTT ATGCGTCTGA 4700
CAAATACCTG AAAATCTGTA TGAGTCAGGT CTTTGAGATA ATGAGGTTCA 4750
TAGAGCCAGG CCTATTCTCA TACTATAGTG GAGAGAAATA ATGAAAATAA 4800
ACTAACAAGC AAAGAGTGTT TTCAGATAGT CCCAAGTCTG ACAGGAAAAA 4850
GAGCAGGCAA AGAAGGCAGT GATGTGGGTG TGGTCGTCTC TGAGAGAGTG 4900
ATGTTTACGC AGAACTGAAG GAGAGAGTCT CGCAAGATGA GAGGCCACAG 4950
GCATTAGAGG CAGAAGGGAC AGGCAGGCAC TGAGGGCCAG TGGCAGGGTC 5000
AGCCTAGGGA CATGGAACCA CCACCATGAG GCCATCGTGT CCGGAGCAAG 5050
TCAGTGCAGA GCAGCAACAG TGAATGTCGC GATGTTCTAA TGATGACAGG 5100
AGACCTTCGG GCTGCAGCTC TAATGTGAGG TGTGGAAATG GGTCATAGAG 5150
AATGAGTGAG CTGGGGAAAA GTCAGCGAGG GGGCCTCTGA GTGATGGGTC 5200
ACACAAGTGA GGGTAGTGCC TCTGAGTGAG GACTCACACA GTGGGGGAGG 5250
CGTTCTGAGT GATGGCTCCC ATGCTGCAGG TGAGCACTGT CAATGAGTTG 5300
GCACTTGAAC CGGACAGGTC TCACTTCATC CTGCTGCTCC AGGCTGGAGC 5350
TACTGCACAG GTTGACTCCT CTCTCTGTAC ATCATCTAAG TAATTGCACG 5400
GCCCGTGGGT CATCTGTTCA AAGTCAGAAA ATAAGGACAG TGTCTTGGGA 5450
CTTAGAAGTA GCAGATGAAA AAACAAATTT AACAATGCTT TTATGGTTTT 5500
AATTTAAAAA AAAAAATTAC ATGTCTACCT ATGTATCTGT GTGTGAGTGT 5550
GTGCATGTGA ATTTAGGGGC CCCTGGAAGA ACAGTGCAAG TTTTTAACCT 5600
CTAGACCTGC ACTTGACAAT ATTGGGGGCG GGAGGGCGGG TTTCTCTGTG 5650
TAGCCCTGGC TGCTCTGGGA CTCACTCTGT AGACCAGGCT GGCCTTGACT 5700

Fig 10P

```
CATAGATCCA CCTGCCTCTG CCTTCTGAGT GCTGGGACCA AAGGTGTGCA    5750
CTACCATGCC CAGCCGACAT ACATTTTTAA AAAAGATTTA TTTTATGTAT    5800
ATGAGTACAT TGTCACTGTC TTCAGACACA CTGGAAGAGT GCATCAGATC    5850
TCATTATAGA TGGTTGTGAG CCACCATGTG GTTGCTGGGA ATTGAACTCC    5900
GGATCTCCAG AAAAGCAGTC AGTGCTCTTA ACTACTGAAC CACCTCTCCA    5950
GCCCAACAAC ACATTTTGAA CAACTCTGTT GAGATGAAAC TCACAGTCAA    6000
TCCATATAAA GTATTCATGT CAACTGAAAG AGTCACGTGA CTGTCACCAC    6050
AAAAGTTCAG AATGTTTTTG CCAGCCCTTT CAGAACATAA GCAAAACCTT    6100
ATGTCCATTA GGGTTCCAAA TCCTTTCCTC CTACCCCAAG AAACCACAGC    6150
TGTCTGTCTT CAACTAGTTT AGATGCTTCA GTTGTGTGGT CAGATGTGGG    6200
CATGCTGAAC ACGGTCCTCT GTGACTGGCT TCTTTTGCTA AGCATGGTTT    6250
TGTTTTGTTT TGTTTTGTTT TTAAGTTTAT CAAGACAGGT TTTCTCTGTA    6300
TAGTTCTGGC CGTCCTGGAA CTCACTCTGT AGACCAGGCT GGCCTCCAAC    6350
TCAGAAATCC ACCTGCCTCT GCCTCGCTAA GCATGCTTTT AAAGTTCATC    6400
TATGTCGGAG CATTCTTCTT AAAAGCTGGT TAATATTCTG TAACGTGGAT    6450
ATAAAATAAA CCTTATTCGT TCCTGGTTGA CATGAGGGTG TTTATCTTTT    6500
TGAGGGGTGG GTCTATCAGG AATAATCCTG TCGGCTTTTT TGAGATGAGG    6550
TGCCATTGCA TTGTCTTGTC CAGTTTTGAA ATGGCAGACT CGGGCATTCT    6600
TCCTGACCTC AGTATATACA TTGACGGAAC ATTATGAAGT CAAAAATACT    6650
GTATCTTGTA GGGAGCTTTA GACCTTTCCA GGTTAGGTAC ACCATTTTAC    6700
ATGATGGTGA TGATCATGTA TGTATCAGGC AGGTTGGTAG GTATGTGTGT    6750
GTGTGTGAGA GAGTGTGTGT GTGTGTGTGT GTGGTCTATG TGTGTGAGTG    6800
TGTGGTCTAT GTGTGTGAGT GTGTGTGTGT GAGTGTGTGT GTGTGTGTGT    6850
GTGTATTGAA CCCAGGGCCT AAAGCATACT GAACCACTGT TCTATCACTG    6900
AACTACACCT CTGACTTCAT GATGGTAATA TGTTAATTGA CGTCCTTTGT    6950
GGTAAGATTT TGAAAGCAGC ACAGATTGAA AATAAGAAAG CTTCAAATAC    7000
AGAAGCCAGT TCCCAGCAGG AACTGGGAGG GCGGCTGGTA AGACCAATCC    7050
AGTACCGGAC ATCCCAGAGT TGAGACACGC ACACACACAC ACACAGTCCC    7100
TATGGAGGGA GCCAGGACTC CCCTTCCCAC CCCAACCCCA ACACATACCC    7150
AGGCAGTCCT GTGCAAGTAC AAGGTCAGCC TAACACCAGG TCTGCACACA    7200
GCCTGTCCAG GCTCCGCACA GCGAGGGTCA CGATCCTCGT CTATGGAGGC    7250
GAAAACTGCC TCAGAGACAG TAGAGTGCTG AGCTGAGGAG TGTGTGGGGC    7300
ACCATGCTGA TCTCTCCAAA TCAGGATGCT GCCAAGGGTG GCAGACTCAA    7350
GCCCTCCTGA CTCCTGCCTC TTCCACCTTA GCCCCAGGCT ATTTATTGCA    7400
AGGATGTCCT GGACATTGAA CAGTTCTCTA CAGTTAAAGG TGTGGATCTG    7450
GAGCCCACAG ACCAAGACTT CTACCAGAAG TTTGCCACAG GCAGTGTGTC    7500
CATCCCCTGG CAGAATGAGG TAGGGATTAT CCTGTGACTG GGATGAGGTC    7550
CAGGTAGGGC ACCCCCAGGG CCAACTCTCA CCGCTTCCTG TTCCTGGGTA    7600
GATGGTGGAG ACCGAGTGCT TCCAGGAACT CAATGTCTTT GGGCTGGATG    7650
GGTCTGTTCC CCCAGACCTG GACTGGAAGG GCCAGCCCAC TGCACCCCCC    7700
AAGAAGGGAT TGCTACAGAG ACTCTTCAGT CGCCAAGTAA GTCCTAGCAG    7750
TGTCTGCCCT GGGTCCCACC TGCTCCACTG GGAGAGTGGG TGGGAGGCAG    7800
AGCTGGTGCA TGCCTGCCCC GGGAGTGGGC ATCTTCCTGT GGTGCCCAAG    7850
GTGCCTCATT CCTGCCTGTC CCCCCTGACC CCCACGCCTG GCTGGTCTCA    7900
TGGCCTCTTT TCTCTTTCCA GAGGTGAGCA GTGTGGGCTC CCCATGGGTT    7950
GGCCTTGCTG CCTGACTGTG GGGAGCCCCA GGCAGCCCAT CCGAGGCAGA    8000
GGCGAGATGT AGGAGAAAGA AGTCTGGTGT CCACCTTACC CGCTCCTTCC    8050
CTGCCGCTTG CCTCCCTGCA CCCTGACCCC TTCAAGCTGC TAACCTTGCT    8100
TAGCAACTGT CTCCTTGTGC CAGAGTCTGT CCAGGGGGAT GGACAGAGGC    8150
CTTGCTCTGA CAGATCGAGT CGACGCCCTA TAGTGAGTCG TATTAGAGCT    8200
CGCGGCCGC                                                 8209
```

Fig 10Q

SEQ ID No: 3
5'-GTCAGGTTTGGAACACCAGTCCTG
RTP756

SEQ ID No: 4
5'-TCTTCAGGTGGGCGCCACTGCAAG
RTP1202

SEQ ID No: 5
5'-AGTGCCTACTGGGTTCAGGCATGGA
RTP1203

Fig 10R

Human GRK6A splice variant
SEQ ID No: 6
atggagct cgagaacatc gtagcgaaca cggtgctact caaggcccgg gaaggtggcg
gtggaaatcg caaaggcaaa agcaagaaat ggcggcagat gctccagttc cctcacatca
gccagtgcga agagctgcgg ctcagcctcg agcgtgacta tcacagcctg tgcgagcggc
acgccattgg gcgcctgctg ttccgagagt tctgtgccac gaggccggag ctgagccgct
gcgtcgcctt cctggatggg gtggccgagt atgaagtgac cccggatgac aagcggaagg
catgtgggcg gcacgtaacg cagaattttc tgagccacac gggtcctgac ctcatccctg
aggtcccccg gcagctggtg acgaactgca cccagcggct ggagcagggt ccctgcaaag
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cagtgaccaa aaacaccttc aggcaatacc gagtcctggg caaaggtggc tttggggagg
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agcggatcaa gaagcggaaa ggggaggcca tggcgctgaa cgagaagcag atcctggaga
aagtgaacag taggtttgta gtgagcttgg cctacgccta tgagaccaag gacgcgctgt
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aggacctgca ccgggagcgc atcgtgtaca gggacctgaa gcccgagaac atcttgctgg
atgaccacgg ccacatccgc atctctgacc tgggactagc tgtgcatgtg cccgagggcc
agaccatcaa agggcgtgtg ggcaccgtgg gttacatggc tccggaggtg gtgaagaatg
aacggtacac gttcagccct gactggtggg cgctcggctg cctcctgtac gagatgatcg
caggccagtc gccccttccag cagaggaaga agaagatcaa gcgggaggag gtggagcggc
tggtgaagga ggtccccgag gagtattccg agcgcttttc ccccgcaggcc cgctcacttt
gctcacagct cctctgcaag gaccctgccg aacgcctggg gtgtcgtggg ggcagtgccc
gcgaggtgaa ggagcacccc ctctttaaga agctgaactt caagcggctg ggagctggca
tgctggagcc gccgttcaag cctgaccccc aggccattta ctgcaaggat gttctggaca
ttgaacagtt ctctacggtc aagggcgtgg agctggagcc taccgaccag gacttctacc
agaagtttgc cacaggcagt gtgcccatcc cctggcagaa cgagatggtg gagaccgagt
gcttccaaga gctgaatgtc tttgggctgg atggctcagt tcccccagac ctggactgga
agggccagcc acctgcacct cctaaaaagg gactgctgca gagactcttc agtcgccaag
attgctgtgg aaactgcagc gacagcgagg aagagctccc caccccgcctc tag

SEQ ID No: 7
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERHAIGRLLFREFCATRPELSRCVAFLDGVAEYEVTPDDKRKACGRHV
TQNFLSHTGPDLIPEVPRQLVTNCTQRLEQGPCKDLFQELTRLTHEYLSVAPFA
DYLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGK
MYACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLV
LTLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPE
NILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVKNERYTFSPDW
WALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVPEEYSERFSPQARSLCS
QLLCKDPAERLGCRGGSAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYC
KDVLDIEQFSTVKGVELEPTDQDFYQKFATGSVPIPWQNEMVETECFQELNVF
GLDGSVPPDLDWKGQPPAPPKKGLLQRLFSRQDCCGNCSDSEEELPTRL

Fig 10S

Human GRK6B splice variant
SEQ ID No: 8
atggagc tcgagaacat cgtagcgaac acggtgctac tcaaggcccg ggaaggtggc
ggtggaaatc gcaaaggcaa aagcaagaaa tggcggcaga tgctccagtt ccctcacatc
agccagtgcg aagagctgcg gctcagcctc gagcgtgact atcacagcct gtgcgagcgg
cagcccattg ggcgcctgct gttccgagag ttctgtgcca cgaggccgga gctgagccgc
tgcgtcgcct tcctggatgg ggtggccgag tatgaagtga ccccggatga caagcggaag
gcatgtgggc ggcagctaac gcagaatttt ctgagccaca cgggtcctga cctcatccct
gaggtccccc ggcagctggt gacgaactgc acccagcggc tggagcaggg tccctgcaaa
gaccttttcc aggaactcac ccggctgacc cacgagtacc tgagcgtggc cccttttgcc
gactacctcg acagcatcta cttcaaccgt ttcctgcagt ggaagtggct ggaaaggcag
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gtgtgcgcct gccaggtgcg ggccacaggt aagatgtatg cctgcaagaa gctagagaaa
aagcggatca agaagcggaa aggggaggcc atggcgctga acgagaagca gatcctggag
aaagtgaaca gtaggtttgt agtgagcttg gcctacgcct atgagaccaa ggacgcgctg
tgcctggtgc tgacactgat gaacgggggc gacctcaagt tccacatcta ccacatgggc
caggctggct tccccgaagc gcgggccgtc ttctacgccg ccgagatctg ctgtggcctg
gaggacctgc accgggagcg catcgtgtac agggacctga agcccgagaa catcttgctg
gatgaccacg gccacatccg catctctgac ctgggactag ctgtgcatgt gccccgagggc
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gcaggccagt cgcccttcca gcagaggaag aagaagatca agcgggagga ggtggagcgg
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tgctcacagc tcctctgcaa ggaccctgcc gaacgcctgg ggtgtcgtgg gggcagtgcc
cgcgaggtga aggagcaccc cctctttaag aagctgaact tcaagcggct gggagctggc
atgctggagc cgccgttcaa gcctgacccc caggccattt actgcaagga tgttctggac
attgaacagt tctctacggt caagggcgtg gagctggagc ctaccgacca ggacttctac
cagaagtttg ccacaggcag tgtgcccatc ccctggcaga acgagatggt ggagaccgag
tgcttccaag agctgaatgt ctttgggctg gatggctcag ttcccccaga cctggactgg
aagggccagc cacctgcacc tcctaaaaag ggactgctgc agagactctt cagtcgccaa
aggattgctg tggaaactgc agcgacagcg aggaagagct ccccacccgc ctctagcccc
cagcccgagg cccccaccag cagttggcgg tag

SEQ ID No: 9
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERQPIGRLLFREFCATRPELSRCVAFLDGVAEYEVTPDDKRKACGRQL
TQNFLSHTGPDLIPEVPRQLVTNCTQRLEQGPCKDLFQELTRLTHEYLSVAPFA
DYLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGK
MYACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLV
LTLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPE
NILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVKNERYTFSPDW
WALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVPEEYSERFSPQARSLCS
QLLCKDPAERLGCRGGSAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYC
KDVLDIEQFSTVKGVELEPTDQDFYQKFATGSVPIPWQNEMVETECFQELNVF
GLDGSVPPDLDWKGQPPAPPKKGLLQRLFSRQRIAVETAATARKSSPPASSPQP
EAPTSSWR

Fig 10T

Human GRK6C splice variant
SEQ ID No: 10
atggagc tcgagaacat cgtagcgaac acggtgctac tcaaggcccg ggaaggtggc
ggtggaaatc gcaaaggcaa aagcaagaaa tggcggcaga tgctccagtt ccctcacatc
agccagtgcg aagagctgcg gctcagcctc gagcgtgact atcacagcct gtgcgagcgg
cagcccattg ggcgcctgct gttccgagag ttctgtgcca cgaggccgga gctgagccgc
tgcgtcgcct tcctggatgg ggtggccgag tatgaagtga ccccggatga caagcggaag
gcatgtgggc ggcagctaac gcagaatttt ctgagccaca cgggtcctga cctcatccct
gaggtccccc ggcagctggt gacgaactgc acccagcggc tggagcaggg tccctgcaaa
gaccttttcc aggaactcac ccggctgacc cacgagtacc tgagcgtggc cccttttgcc
gactacctcg acagcatcta cttcaaccgt ttcctgcagt ggaagtggct ggaaaggcag
ccagtgacca aaaacacctt caggcaatac cgagtcctgg gcaaaggtgg ctttggggag
gtgtgcgcct gccaggtgcg ggccacaggt aagatgtatg cctgcaagaa gctagagaaa
aagcggatca agaagcggaa aggggaggcc atggcgctga acgagaagca gatcctggag
aaagtgaaca gtaggtttgt agtgagcttg gcctacgcct atgagaccaa ggacgcgctg
tgcctggtgc tgacactgat gaacgggggc gacctcaagt tccacatcta ccacatgggc
caggctggct tccccgaagc gcgggccgtc ttctacgccg ccgagatctg ctgtggcctg
gaggacctgc accgggagcg catcgtgtac agggacctga agcccgagaa catcttgctg
gatgaccacg gccacatccg catctctgac ctgggactag ctgtgcatgt gccccgagggc
cagaccatca aagggcgtgt gggcaccgtg ggttacatgg ctccggaggt ggtgaagaat
gaacggtaca cgttcagccc tgactggtgg gcgctcggct gcctcctgta cgagatgatc
gcaggccagt cgccсttcca gcagaggaag aagaagatca agcgggagga ggtggagcgg
ctggtgaagg aggtccccga ggagtattcc gagcgctttt ccccgcaggc ccgctcactt
tgctcacagc tcctctgcaa ggaccctgcc gaacgcctgg ggtgtcgtgg gggcagtgcc
cgcgaggtga aggagcaccc cctctttaag aagctgaact tcaagcggct gggagctggc
atgctggagc cgccgttcaa gcctgacccc caggccattt actgcaagga tgttctggac
attgaacagt tctctacggt caagggcgtg gagctggagc ctaccgacca ggacttctac
cagaagtttg ccacaggcag tgtgcccatc ccctggcaga acgagatggt ggagaccgag
tgcttccaag agctgaatgt ctttgggctg gatggctcag ttcccccaga cctggactgg
aagggccagc cacctgcacc tcctaaaaag ggactgctgc agagactctt cagtcgccaa
aggtga

SEQ ID No: 11
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERQPIGRLLFREFCATRPELSRCVAFLDGVAEYEVTPDDKRKACGRQL
TQNFLSHTGPDLIPEVPRQLVTNCTQRLEQGPCKDLFQELTRLTHEYLSVAPFA
DYLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGK
MYACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLV
LTLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPE
NILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVKNERYTFSPDW
WALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVPEEYSERFSPQARSLCS
QLLCKDPAERLGCRGGSAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYC
KDVLDIEQFSTVKGVELEPTDQDFYQKFATGSVPIPWQNEMVETECFQELNVF
GLDGSVPPDLDWKGQPPAPPKKGLLQRLFSRQR

Fig 10U

Mouse GRK6A splice variant
SEQ ID No: 12
at ggagctcgag
aacatcgtag cgaacacggt gctactcaag gcccgggaag gtggtggcgg gaatcgcaaa
ggcaagagca agaaatggcg ccagatgctg cagttccccc atatcagcca gtgtgaggag
cttcgactca gccttgagcg tgactaccac agcctatgtg agcgccagcc cattgggcgc
ctgttatttc gtgagttctg tgctacgaga cctgagctga cccggtgtac tgccttcctg
gatggggtgt ctgaatatga ggtgacccct gatgagaagc ggaaagcatg tgggcgccga
ctaatgcaga actttctgag ccacacgggt cctgacctca tccctgaagt tccacggcag
ctggtgagta actgtgccca gcggctagag cagggaccct gcaaagacct cttccaggag
ctgacccggc tgacccacga gtacctgagc acggcccctt ttgccgacta cctcgacagc
atctacttca accgttttct gcagtggaag tggctggaaa ggcaaccagt gaccaaaaac
accttcaggc agtaccgagt cctgggcaaa ggtggctttg gggaggtatg tgcctgccag
gtgcgggcaa caggcaagat gtacgcatgc aagaaactgg aaaagaagcg gataaagaag
cgaaaggggg aggccatggc tctcaacgag aaacagatct tggagaaagt gaacagtagg
tttgtagtga gcttagccta cgcctatgag accaaggatg cactgtgcct ggtgctgaca
ttgatgaatg gaggtgacct aaagttccac atctaccaca tgggccaggc tggctttcct
gaagcacgtg ctgtcttcta tgctgctgag atctgctgtg gcctggaaga cctgcaccgg
gaacgcattg tgtacaggga tctaaagcca gagaatatcc ttctggatga ccatggccac
attcggatct cggacctggg actggccgtg catgtgcctg agggccagac catcaaaggc
cgtgtgggca ctgtgggcta catggctcca gaggtggtga ggaatgagcg ctacacgttc
agtcctgact ggtgggcgct aggctgcctc ctgtacgaga tgatcgcagg tcagtcgccc
ttccagcaga ggaagaagaa gataaagcgc gaagaggtgg agcggctggt caaggaagtg
gccgaggagt acacagaccg cttttcctca caggcgcgct cactctgttc tcagcttctt
agcaaggacc ctgctgagcg cctggggtgt cgtggaggtg gcgcccgtga ggtaaaggag
caccccccttt tcaagaaact gaatttcaag cggctgggag ctggcatgct agagccacct
tttaagcctg atccccaggc tatttattgc aaggatgtcc tggacattga acagttctct
acagttaaag gtgtggatct ggagcccaca gaccaagact tctaccagaa gtttgccaca
ggcagtgtgt ccatcccctg gcagaatgag atggtggaga ccgagtgttt ccaggaactc
aatgtctttg ggctggatgg gtctgttccc ccagacctgg actggaaggg ccagcccact
gcaccccccca agaagggatt gctacagaga ctcttcagtc gccaagattg ctgtgggaac
tgcagcgaca gtgaggaaga gctccccacc cgcctctag

SEQ ID No: 13
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERQPIGRLLFREFCATRPELTRCTAFLDGVSEYEVTPDEKRKACGRRLM
QNFLSHTGPDLIPEVPRQLVSNCAQRLEQGPCKDLFQELTRLTHEYLSTAPFAD
YLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGKM
YACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLVL
TLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPEN
ILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVRNERYTFSPDWW
ALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVAEEYTDRFSSQARSLCSQ
LLSKDPAERLGCRGGGAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYCK
DVLDIEQFSTVKGVDLEPTDQDFYQKFATGSVSIPWQNEMVETECFQELNVFG
LDGSVPPDLDWKGQPTAPPKKGLLQRLFSRQDCCGNCSDSEEELPTRL

Fig 10V

Mouse GRK6B splice variant
SEQ ID No: 14
at ggagctcgag
aacatcgtag cgaacacggt gctactcaag gcccgggaag gtggtggcgg gaatcgcaaa
ggcaagagca agaaatggcg ccagatgctg cagttccccc atatcagcca gtgtgaggag
cttcgactca gccttgagcg tgactaccac agcctatgtg agcgccagcc cattgggcgc
ctgttatttc gtgagttctg tgctacgaga cctgagctga cccggtgtac tgccttcctg
gatggggtgt ctgaatatga ggtgacccct gatgagaagc ggaaagcatg tgggcgccga
ctaatgcaga actttctgag ccacacgggt cctgacctca tccctgaagt tccacggcag
ctggtgagta actgtgccca gcggctagag cagggaccct gcaaagacct cttccaggag
ctgacccggc tgacccacga gtacctgagc acggccccctt ttgccgacta cctcgacagc
atctacttca accgttttct gcagtggaag tggctggaaa ggcaaccagt gaccaaaaac
accttcaggc agtaccgagt cctgggcaaa ggtggctttg ggggaggtatg tgcctgccag
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ttgatgaatg gaggtgacct aaagttccac atctaccaca tgggccaggc tggctttcct
gaagcacgtg ctgtcttcta tgctgctgag atctgctgtg gcctggaaga cctgcaccgg
gaacgcattg tgtacaggga tctaaagcca gagaatatcc ttctggatga ccatggccac
attcggatct cggacctggg actggccgtg catgtgcctg agggccagac catcaaaggc
cgtgtgggca ctgtgggcta catggctcca gaggtggtga ggaatgagcg ctacacgttc
agtcctgact ggtgggcgct aggctgcctc ctgtacgaga tgatcgcagg tcagtcgccc
ttccagcaga ggaagaagaa gataaagcgc gaagaggtgg agcggctggt caaggaagtg
gccgaggagt acacagaccg cttttcctca caggcgcgct cactctgttc tcagcttctt
agcaaggacc ctgctgagcg cctgggggtgt cgtggaggtg gcgcccgtga ggtaaaggag
cacccccttt tcaagaaact gaatttcaag cggctgggag ctggcatgct agagccacct
tttaagcctg atccccaggc tatttattgc aaggatgtcc tggacattga acagttctct
acagttaaag gtgtggatct ggagcccaca gaccaagact tctaccagaa gtttgccaca
ggcagtgtgt ccatcccctg gcagaatgag atggtggaga ccgagtgttt ccaggaactc
aatgtctttg ggctggatgg gtctgttccc ccagacctgg actggaaggg ccagcccact
gcacccccca agaagggatt gctacagaga ctcttcagtc gccaaaggat tgctgtggga
actgcagcga cagtgaggaa gagctcccca cccgcctcta gcccccaggc cgaggccccc
accggcggtt ggcggtag

SEQ ID No: 15
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERQPIGRLLFREFCATRPELTRCTAFLDGVSEYEVTPDEKRKACGRRLM
QNFLSHTGPDLIPEVPRQLVSNCAQRLEQGPCKDLFQELTRLTHEYLSTAPFAD
YLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGKM
YACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLVL
TLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPEN
ILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVRNERYTFSPDWW
ALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVAEEYTDRFSSQARSLCSQ
LLSKDPAERLGCRGGGAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYCK
DVLDIEQFSTVKGVDLEPTDQDFYQKFATGSVSIPWQNEMVETECFQELNVFG
LDGSVPPDLDWKGQPTAPPKKGLLQRLFSRQRIAVGTAATVRKSSPPASSPQA
EAPTGGWR

Fig 10W

Mouse GRK6C splice variant
SEQ ID No: 16
at ggagctcgag
aacatcgtag cgaacacggt gctactcaag gcccgggaag gtggtggcgg gaatcgcaaa
ggcaagagca agaaatggcg ccagatgctg cagttccccc atatcagcca gtgtgaggag
cttcgactca gccttgagcg tgactaccac agcctatgtg agcgccagcc cattgggcgc
ctgttatttc gtgagttctg tgctacgaga cctgagctga cccggtgtac tgccttcctg
gatggggtgt ctgaatatga ggtgacccct gatgagaagc ggaaagcatg tgggcgccga
ctaatgcaga actttctgag ccacacgggt cctgacctca tccctgaagt tccacggcag
ctggtgagta actgtgccca gcggctagag cagggaccct gcaaagacct cttccaggag
ctgacccggc tgacccacga gtacctgagc acggcccctt ttgccgacta cctcgacagc
atctacttca accgttttct gcagtggaag tggctggaaa ggcaaccagt gaccaaaaac
accttcaggc agtaccgagt cctgggcaaa ggtggctttg gggaggtatg tgcctgccag
gtgcgggcaa caggcaagat gtacgcatgc aagaaactgg aaaagaagcg gataaagaag
cgaaaggggg aggccatggc tctcaacgag aaacagatct tggagaaagt gaacagtagg
tttgtagtga gcttagccta cgcctatgag accaaggatg cactgtgcct ggtgctgaca
ttgatgaatg gaggtgacct aaagttccac atctaccaca tgggccaggc tggctttcct
gaagcacgtg ctgtcttcta tgctgctgag atctgctgtg gcctggaaga cctgcaccgg
gaacgcattg tgtacaggga tctaaagcca gagaatatcc ttctggatga ccatggccac
attcggatct cggacctggg actggccgtg catgtgcctg agggccagac catcaaaggc
cgtgtgggca ctgtgggcta catggctcca gaggtggtga ggaatgagcg ctacacgttc
agtcctgact ggtgggcgct aggctgcctc ctgtacgaga tgatcgcagg tcagtcgccc
ttccagcaga ggaagaagaa gataaagcgc gaagaggtgg agcggctggt caaggaagtg
gccgaggagt acacagaccg cttttcctca caggcgcgct cactctgttc tcagcttctt
agcaaggacc ctgctgagcg cctggggtgt cgtggaggtg gcgcccgtga ggtaaaggag
caccccctttt tcaagaaact gaatttcaag cggctgggag ctggcatgct agagccacct
tttaagcctg atccccaggc tatttattgc aaggatgtcc tggacattga acagttctct
acagttaaag gtgtggatct ggagcccaca gaccaagact tctaccagaa gtttgccaca
ggcagtgtgt ccatcccctg gcagaatgag atggtggaga ccgagtgttt ccaggaactc
aatgtctttg ggctggatgg gtctgttccc ccagacctgg actggaaggg ccagcccact
gcaccccccca agaagggatt gctacagaga ctcttcagtc gccaaaggtg a

SEQ ID No: 17
MELENIVANTVLLKAREGGGGNRKGKSKKWRQMLQFPHISQCEELRLSLERD
YHSLCERQPIGRLLFREFCATRPELTRCTAFLDGVSEYEVTPDEKRKACGRRLM
QNFLSHTGPDLIPEVPRQLVSNCAQRLEQGPCKDLFQELTRLTHEYLSTAPFAD
YLDSIYFNRFLQWKWLERQPVTKNTFRQYRVLGKGGFGEVCACQVRATGKM
YACKKLEKKRIKKRKGEAMALNEKQILEKVNSRFVVSLAYAYETKDALCLVL
TLMNGGDLKFHIYHMGQAGFPEARAVFYAAEICCGLEDLHRERIVYRDLKPEN
ILLDDHGHIRISDLGLAVHVPEGQTIKGRVGTVGYMAPEVVRNERYTFSPDWW
ALGCLLYEMIAGQSPFQQRKKKIKREEVERLVKEVAEEYTDRFSSQARSLCSQ
LLSKDPAERLGCRGGGAREVKEHPLFKKLNFKRLGAGMLEPPFKPDPQAIYCK
DVLDIEQFSTVKGVDLEPTDQDFYQKFATGSVSIPWQNEMVETECFQELNVFG
LDGSVPPDLDWKGQPTAPPKKGLLQRLFSRQR

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/20838

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01K 67/00; G01N 33/00; C12N 9/12
US CL : 800/3, 8, 21; 435/194
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/3, 8, 21; 435/194

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
provisional US application

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, medline, PALM for inventor search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,444,456 A (WALKE et al.) 03 September 2002 (03.09.2002) see entire reference.	1-38
Y	US 6,255,069 B1 (BENOVIC et al.) 03 July 2001 (03.07.2001), see entire reference.	1-38
Y	US 5,591,618 A (CHANTRY et al.) 07 January 1997 (07.01.1997), see entire reference.	1-38
X — Y	FONG et al. Defective lymphocyte chemotaxis in beta arrestin2 and GRK6-deficient mice. Proc. Natl. Acad. Sci. USA. 28 May 2002, Vol. 99, No. 11, pages 7478-83, see entire reference.	1-12, 37, 38 — 13-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

Published:

— *with international search report*

— *with amended claims*

Date of publication of the amended claims: 15 April 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/004451 A1

(54) **Title:** METHODS OF SCREENING COMPOUNDS FOR GRK6 MODULATING ACTIVITY

(57) **Abstract:** The present invention relates to methods of treating disease by altering G protein coupled receptor kinase (GRK) 6. This may be done by altering the expression or activity of the protein, for example. The present invention may be used for disease diagnosis, by detecting the expression or activity of GRK6. The present invention relates to a GRK6 deficient mouse, GRK6 splice variants, and methods of use. The present invention also relates to methods of identifying compounds that alter GRK6 activity. The present invention relates to disease treatment by altering GRK6 expression or activity.

AMENDED CLAIMS

[Received by the International Bureau on 06 February 2004 (06.02.04):
original claims 1 - 38 replaced by amended claims 1-31 (3 pages)]

1. A method of screening for modulators of GRK6-associated desensitization comprising: (a) providing a cell comprising a GRK6 and a GPCR; (b) contacting said cell with a candidate modulator; and (c) monitoring said cell for GRK6-associated desensitization.

2. The method of claim 1, wherein the monitoring comprises determining the cellular distribution of the GRK6, GPCR, or arrestin.

3. A method for identifying compounds that modulate GRK6 comprising the steps of:
(a) providing a cell comprising GRK6, a GPCR, and an arrestin, and wherein at least one of said molecules is detectably labeled;
(b) exposing the cell to the compound(s);
(c) determining the cellular distribution of the GRK6, GPCR, or arrestin;
(d) comparing the cellular distribution of the GRK6, GPCR, or arrestin in the presence of the compound(s) to the cellular distribution of the GRK6, GPCR, or arrestin in the absence of the compound(s); and
(e) correlating a difference between (1) the cellular distribution of the GRK6, GPCR, or arrestin in the presence of the compound(s) to (2) the cellular distribution of the GRK6, GPCR, or arrestin in the absence of the compound(s) to modulation of GRK6 activity.

4. The method of claim 4, wherein the GRK6 is overexpressed.

5. The method of claim 3, wherein the labeled molecule is localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles or endosomes.

6. The method of claim 3, wherein the detectable molecule is a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group.

7. The method of claim 3, wherein the molecule is detectably labeled due to its interaction with another molecule, which may be detectably labeled.

8. A method for inhibiting desensitization of the dopamine receptor in cell comprising contacting the cell with a compound that decreases GRK6 activity or the expression of a nucleic acid encoding GRK6.

9. The method of claim 8, wherein the compound is an antisense oligonucleotide.

10. The method of claim 9, wherein the antisense oligonucleotide inhibits expression of a nucleic acid encoding GRK6.

11. A method for treating diseases involving the dopamine receptor, wherein the effectiveness of endogenous dopamine is increased by altering GRK6 activity or expression.

12. The method of claim 11, wherein the disease is Parkinson's, schizophrenia, Tourette Syndrome, depression, or drug-addiction.

13. A method of modulating desensitization of a dopamine receptor in a cell, comprising:
(a) providing a cell expressing a dopamine receptor and a G protein coupled receptor kinase (GRK);
(b) modulating the activity of the GRK; and
(c) exposing said cell to an agonist.

14. The method of claim 13, wherein the GRK is GRK6.

15. The method of claim 14, wherein the expression of GRK6 is increased.

16. The method of claim 14, wherein the expression of GRK6 is decreased

17. The method of claim 14, wherein the activity of GRK6 is increased.

18. The method of claim 14, wherein the activity of GRK6 is decreased.

19. A method of treating a disease by modulating desensitization of a dop receptor in a host cell, comprising: (a) providing a compound which modulates the expression or activity of a GRK6; and (b) administering said compound to a host.

20. The method of claim 19, wherein said method comprises concurrent of the compound that modulates expression or activity of a GRK6 with a compound that modulates a G-protein coupled receptor.

21. A nucleic acid selected from the group consisting of SEQ ID Nos: 1-3.

22. A nucleic acid selected from the group consisting of SEQ ID Nos: 4-5.

23. A vector comprising the nucleic acid of SEQ ID Nos:1-3.

24. The vector of claim 23, wherein the nucleic acid is flanked by loxP sites.

25. A host cell comprising the nucleic acid of SEQ ID No:19.

26. An isolated immunoglobulin which recognizes and binds to a GRK, or fragment thereof.

27. The immunoglobulin of claim 26, wherein the GRK6 is GRK6a, GRK6b, GRK6c, or GRK6d.

28. The immunoglobulin of claim 26, wherein the GRK fragment has the sequence of SEQ ID No. 3.

29. The immunoglobulin of claim 26, wherein the antibody fragment is Fab, Fab', F(ab')2, F(v), and scFv.

30. A method of detecting GRK6 in a biological sample, comprising:
(a) exposing the biological sample to an immunoglobulin of claim 26; and
(b) determine whether the immunoglobulin bound a protein of the biological sample.

31. The method of 30, wherein the binding of the immuno globulin to the protein indicates the presence of or predis-position to a disease.